Programa de Doctorado “Química Ambiental y Fundamental” (RD 1393/2007)

Departamento de Química Analítica. Instituto Universitario de Medio Ambiente

Universidade da Coruña

DETERMINACIÓN DE TRIAZINAS EN MEDIO MARINO

Memoria presentada por

NOELIA RODRÍGUEZ GONZÁLEZ

para optar al grado de Doctora por la Universidade da Coruña con Mención Internacional

Directoras:

Dra. María Elisa Beceiro González

Dra. María José González Castro

A Coruña, diciembre de 2016

UNIVERSIDADE DA CORUÑA

INSTITUTO UNIVERSITARIO DE MEDIO AMBIENTE UNIVERSIDADE DA CORUÑA

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Dra. Dª. PURIFICACIÓN LÓPEZ MAHÍA, Catedrática y Directora del Departamento de Química Analítica de la Universidade da Coruña,

AUTORIZA a **Dª. NOELIA RODRÍGUEZ GONZÁLEZ** a presentar el trabajo titulado "DETERMINACIÓN DE TRIAZINAS EN MEDIO MARINO" para optar al grado de Doctora por la Universidade da Coruña con Mención Internacional.

A Coruña, diciembre de 2016

Dra. Dª. Purificación López Mahía

UNIVERSIDADE DA CORUÑA
Departamento de Química Analítica

Dra. Dª. MARÍA ELISA BECEIRO GONZÁLEZ y **Dra. Dª. MARÍA JOSÉ GONZÁLEZ CASTRO,** Profesoras Titulares del Departamento de Química Analítica de la Universidade da Coruña,

CERTIFICAN

Que la presente Tesis Doctoral titulada "DETERMINACIÓN DE TRIAZINAS EN MEDIO MARINO" se ha realizado bajo su dirección en el Departamento de Química Analítica de la Universidade da Coruña.

Y para que así conste, a los efectos oportunos, firman la presente en A Coruña, a 16 de diciembre de 2016.

Dra. Dª. María Elisa Beceiro González

Dra. Dª. María José González Castro

***PARA TI, MAMÁ**. Mi gemelier.*

Mi madre, hermana y mi mejor amiga. Mi complemento.

*Mi compañera de viaje y de vida. **MI TODO**.*

Para ti, porque te quiero, y en la vida, juntas,

codo a codo, somos mucho más que dos.

Porque sin ti, nada quiero. Porque sin ti, nada soy.

AGRADECIMIENTOS

En primer lugar, quisiera agradecer a todas las personas e instituciones que han colaborado y financiado esta Tesis Doctoral, porque sin su apoyo no habría sido posible llevarla a cabo.

Al Departamento de Química Analítica de la Universidade de A Coruña y al Instituto Universitario de Medio Ambiente (IUMA), por haberme dejado formar parte del gran equipo que han creado haciéndome sentir una más entre ellos y posibilitando la realización de esta Tesis. En especial a mis directoras, Dña. Mª Elisa Beceiro González y Dña. Mª José González Castro, por depositar su confianza en mí y apoyarme en este proyecto.

A la Univerdidade de A Coruña, la Xunta de Galicia y el Campus de Excelencia Internacional Campus do Mar, por haberme concedido el privilegio de disfrutar de ayudas y contratos para elaborar el presente trabajo.

Os meus agradecimentos ao Instituto da Água da Região Norte (IAREN) em Matosinhos, Porto (Portugal) na pessoa da sua Directora, Dra. Maria Fátima Alpendurada, pelo magnífico acolhimento e oportunidade de fazer parte do seu grupo de trabalho, bem como pela confiança e bondade demonstradas, ciente do que eu precisava e sempre dísponivel, quer como Directora de um grupo de alto prestígio internacional, quer a nível pessoal. A todos vós, Marta(s) Vera, Andreia, Teresa..., pela boa disposição que as acompanha no dia-a-dia, e por ensinar-me a vossa maneira de trabalhar. Mas acima de tudo, quero agradecer à Ana Guimarães cada momento que eu passei com ela, tudo o que me ensinou, a confiança e carinho que depositou em mim. Obrigada por fazer com que a minha passagem no IAREN tenha sido tão especial.

A los Servicios de Apoio á Investigación (SAI) de la Universidade de A Coruña, especialmente a la Unidad de Técnicas Cromatográficas, donde he pasado muchos momentos durante estos últimos años y en donde he conocido a Gerardo, quien me ha enseñado tanto sobre espectrometría de masas. Gracias por hacerme siempre un hueco en tu ajustada agenda y por tener tanta paciencia conmigo y ayudarme tantísimo!.

Gracias al Profesor Andrés Martínez Lage y a su grupo de trabajo por permitirme utilizar sus neveras, congeladores, estanterías y por los litros de agua de mar y agua Milli-Q que me habéis proporcionado. Conoceros ha sido todo un placer!. También al Profesor Moisés Canle por cederme material de su grupo de investigación y por la calma que me transmite siempre que habla conmigo.

También quiero agradecer a la Doctora Mª Teresa Rodríguez Blas, coordinadora del Programa de Doctorado de Química Ambiental y Fundamental su dedicación y la implicación que siempre me ha demostrado tener en todo lo que hace ya que se ha movido todo cuanto ha podido para ayudarme a solucionar cada problema. Y sobre todo por su apoyo incondicional en uno de los momentos más difíciles que he vivido desde que estoy en la UDC, el mes de julio de 2015. Gracias por ese congreso que tú hiciste especial donde todos “tus niños” nos sentimos protegidos y arropados por ti. Gracias Mayte!!

Gracias a todos los que formáis parte del grupo QANAP, que aun siendo muchos y tan diferentes, de todos me llevo alguna anécdota y algo nuevo aprendido:

A “los jefes” (Darío, Sol y Puri), por su atención y sus continuos consejos, y las dosis de positivismo que siempre han intentado transmitirnos a los doctorandos, procurando que veamos el futuro un poco más colorido.

A Alatzne, por ser la primera persona de este departamento que me tendió la mano logrando que me aventurase a formar parte del mismo tras mi primera beca de colaboración. Desde que te conocí el primer año de carrera hasta el día de hoy (y ya ha pasado una década) no has dejado de brindarme tu ayuda y apoyo. Por eso, no sólo me has dejado huella como profesora, sino también como persona.

A Jorge, por ayudarme con todas mis dudas, facilitarme las cosas y dejarme sus muestras para mi experimentación!. Eres un profe increíble y especial para mí.

A Maribel, porque no olvido mis inicios en el departamento, estando ella siempre pendiente de que me fuesen bien las cosas y enseñándome cada día algo desconocido para mí.

Gracias también a Andrade, a Isa, a Rosa y a Mari Carmen, por los ratitos de charlas cuando nos encontramos en el laboratorio o en las comidas del departamento, donde me habéis hecho sentir una más entre vosotros. Y por supuesto a Elia, la profesora que más tiempo comparte con nosotros en el laboratorio y a la que tanto cariño he cogido porque nos entiende, anima y aconseja en nuestro día a día.

A las “eternas niñas”, con las que he convivido dentro del laboratorio durante estos años; a Gloria mi compi de HPLC-MS del SAI, a Fany por enseñarme a cambiar la bala de N_2 y echarme una mano con la impresora, a Vero por su alegría y su risa escandalosa y a Carmen porque me transmite la tranquilidad que a mí me falta. Gracias!

A mis compañeros de fatigas… los que comparten mis preocupaciones. María, contigo empecé esta etapa en 2006 en la Licenciatura de Química (y mira si ha pasado tiempo que ni siquiera existe esa titulación…). Entramos juntas en este departamento, en el máster y en la Tesis Doctoral, y por si fuese poco compartimos nuestro problemático HPLC!. A ti, gracias por todo lo que me has ayudado y por ser tan buena amiga. Cuenta conmigo para todo! A Javi por cuidar de mi portátil y por transformar las mañanas de obligaciones en momentos distendidos. Me alegro mucho de haberos conocido tanto a ti como a Cris y espero que esta amistad dure muchos años aunque ahora estemos separados. Y a mi querida tocaya Noe Salgueiro, que aunque nos lleva mucha ventaja, nos va abriendo el camino (además de abrirnos los ojos…) y pone el toque positivo que necesitamos para ver la vida más colorida!. Seguiré pidiéndote consejos allá donde vaya, porque aprender de ti es un placer. Gracias a los cuatro.

A Vero y Rocío, por haber formado parte de nuestro equipo en la realización de sus trabajos de fin de grado y por haberse convertido en más que compañeras para mí. Lo que unió la química que no lo separe nadie.

A mis directoras de tesis, mis jefas!. Elisa, gracias por ver siempre el vaso medio lleno, por encontrar la parte positiva de las cosas y por tirar hacia adelante cuando yo lo veía todo tan

oscuro. Mariajo, gracias por tu constancia, tu paciencia conmigo en lo mucho que me has enseñado, tu sinceridad continua y la manera en la que me has tratado cada día. Con el esfuerzo conjunto de las tres he logrado llegar al final de esta etapa de la que sin duda vosotras formáis parte fundamental y directa. Quiero agradeceros a las dos todo el trabajo que habéis realizado para que esta Tesis se llevase a cabo. Podéis estar seguras de que nunca os olvidaré!.

También quiero agradecerle a Andrés Morado las innumerables veces que me demuestra que aún quedan personas, compañeros y amigos que nunca fallan. Gracias por facilitarme lo que ha estado de tu mano y por tu amabilidad. Te deseo que la vida te dé todo lo que tú nos das a los que te tenemos cerca. Muchas gracias amigo!. Y a Antía Villarino, por ofrecerme su ayuda un en esta última etapa de Tesis y Máster simultáneos, donde hubo días en los que mis horas de sueño nocturnas duraban menos que una pequeña siesta. Es un placer tenerte en mi vida!

A mi "colega de gabinete", Marlene, con la que voy a descubrir los lugares más bonitos de Portugal. Tú fuiste una pieza clave en mi estancia en Matosinhos, por nuestras confidencias, por el frío que pasamos y por las risas que compartimos. Por cada rato en el microondas, los buenos momentos en el comedor y las tardes culturales por el centro de Oporto, y como no, por nuestro estadio, nuestro Dragão!. Te echo de menos compañera!. Y también a Mariluz y Paulo porque me facilitaron la llegada a un nuevo país, y estuvieron pendientes de mí en todo momento. Qué bonitos recuerdos guardo de "mi casa" y cuánto extraño el Pingo Doce!!!

A esa gente que sin formar parte de mi departamento ha hecho que mi vida en la facultad haya sido más agradable. Sobre todo a mi querido profe Fernando Avecilla, porque tus sonrisas valen oro, y verte es siempre motivo de alegría. Con profesores como tú la docencia es mucho más bonita. Gracias por la forma que tienes de llegar a las personas. En mí dejaste una huella imborrable. Tampoco quiero olvidar al personal de la facultad (secretario, conserjes, personal de limpieza o de administración y trabajadores de la cafetería) por haberme tratado como si llevase aquí toda la vida, y haberme hecho sentir como en casa. Gracias a todos.

No puedo nombrar a todas las personas ajenas a esta universidad que han pasado por mi vida durante estos años ayudándome de una manera u otra. AMIGOS que desde La Rúa, Chao das Donas, Benidorm, Cambela, Coruña, Playa América…, me muestran su apoyo y desde el principio confiaron en que podría conseguir este proyecto, incluso en los momentos en los que ni yo misma sabía si podría hacerlo. Es probable que de no haberme sentido tan arropada hubiera abandonado el barco hace meses. Eso es algo que nunca sabré… pero lo que tengo claro es que vosotros me habéis hecho el camino mucho más agradable. No podría nombraros a todos, pero si leéis esto, sabréis quienes sois. Y sobre todo, a esas personas que están a diario, y que no dejan de repetirme que puedo con todo, que siga, que ya falta poco y que no me rinda ahora después de haber luchado tanto. En especial a María no solo por apostar por mí de manera incondicional, y por su apoyo continuo, sino también por cuidar de mi madre cuando yo estoy lejos de ella. Gracias por ser como eres y por estar en nuestras vidas. A los amigos que están lejos pero que siento muy cerca, por haber dejado huella en mi camino: Jona, Marina, Jesús, Elvira, Villa, Pilar G, Miriam, Angelines, Alfredo, Ludi, Carlos… Y especialemte a Kiara, una

niña que me alegra los peores días y me acompaña allí donde voy. Mi ojito derecho. A todos, Gracias!

Y para finalizar, GRACIAS A MI FAMILIA. A los que ya no están y tanto han pedido por mí (mi tío José, mis abuelos, Pepita…) y a los que lo siguen haciendo cada día del año (mi tía Sinda, Pancho y Tere, mi madrina Paz, mi tía Inés…). A mi prima Julia porque para mí es especial y la quiero con todas mis ganas y a mi tío Juan, por demostrarme que para ser feliz solo hay que querer lo que se tiene.

A MI HERMANO, la persona con la que convivo diariamente desde que empecé mi etapa universitaria en la UDC y la persona más culta e inteligente que conozco. Porque aunque estar donde estoy es obra de mi esfuerzo, fue él quien destacando siempre desde pequeño despertó en mí las ganas de intentar estar a su altura. Siempre me dejaste el pabellón muy alto y sin saberlo, tú colaboraste a que llegase hasta aquí. GRACIAS GABRIEL.

A MI PADRE, con el que además de parecido físico comparto carácter y genio. Gracias por aconsejarme aunque no siempre actúe como debería; gracias por cada vez que intentas ayudarme a mejorar a pesar de que no te lo pongo fácil. Sé que me quieres muchísimo y sufres cuando me hacen daño. Papi, ojalá estés tan orgulloso de mí como yo lo estoy de ti, porque no te cambio por ningún otro. Tú has sido mi apoyo en los momentos más duros de mi vida y eso no se olvida. Gracias por estar a mi lado y transmitirme tanto amor, gracias por tu positividad, por creer que siempre conseguiré lo que me proponga y gracias por la infancia y la adolescencia que nos habéis regalado al Gagá y a mí. Por cada verano, por cada Navidad, y por cada sacrificio. Nunca olvides que te admiro hoy y te admiraré siempre. GRACIAS PAPÁ.

AL PILAR DE MI VIDA, MI MADRE. Es imposible expresar en un papel todo lo que necesito agradecerte y lo muchísimo que significas para mí, así que por más que escriba me va a saber a poco, ya que siempre vas a merecer infinitamente más. Quiero que sepas, que cada fracaso de mi vida me duele más por ti que por mí y que cada uno mis éxitos no es solo mío, es obra de las dos, porque todos y cada uno de ellos te los debo a ti, pues eres la persona que desde que me cogió en brazos por primera vez no ha dejado de preocuparse de que todo me vaya bien, la que me protege, me guía, me enseña, me corrige, me cuida, me hace mejorar y vive por y para mí. Tienes que saber que tú eres mi felicidad y que estoy orgullosísima de ser tu hija porque tú eres mucho más que una madre, eres mi mejor amiga, mi compañera de viaje y de vida. Tú eres quien me motiva y pasa noches en vela a mi lado para que no me sienta sola, o acunándome cuando no podía respirar. Eres la base de todo lo que empiezo y la única meta que deseo alcanzar; eres mi principio y mi fin, mi mejor premio, mi motivo de alegría. Gracias por cada sacrificio, por cada caricia, por cada mirada y por cada sonrisa de complicidad; por darme la mano y hacerme sentirlo todo y por ser mi incondicional. Gracias por educarme con tanto amor, por recorrer el mundo a mi lado, por llevarme a mil lugares para que no haya un solo rincón que me falte por descubrir, y sobre todo gracias por compartirlo todo conmigo y regalarme tu tiempo. Porque juntas somos una sola. NO ME FALTES NUNCA. ETERNAMENTE, GRACIAS.

Y ya para terminar, quiero centrarme en los más importantes. Las dos personas con las que empecé esta Tesis, y con las que no voy a poder celebrar el haberlo logrado, MIS PEPÉS. A vosotros os debo mi vida, os lo debo absolutamente todo. Os echo de menos cada noche antes de dormirme y cada día que me despierto. ME HACÉIS FALTA y sois lo único que quiero para volver a ser la niña con más suerte del mundo, como lo he sido todos esos años que os tuve a mi lado. Daría absolutamente todo lo que he conseguido por poder disfrutaros más tiempo. Y es que todavía no entiendo mi vida sin teneros junto a mí, sin tocaros la mano y comeros a besos hasta cansaros de tanto amor. Esta Tesis y cada uno de los proyectos que consiga en la vida deberían llevar también vuestro nombre, porque lo que soy lo habéis logrado vosotros a base de valores y cariño incesante. Y es que en mi niñez no hay un solo recuerdo que no se centre en vosotros. Gracias por haberos sentido tan orgullosos de mí, por presumir de nieta y demostrarme que para los dos soy y seré siempre la mejor. Gracias por dedicar vuestra vida a criarnos, cuidarnos, enseñarnos y querernos. A lo largo de esta Tesis he necesitado muchas veces un abrazo vuestro, o una de esas palabras que lo resuelven todo y que solo vosotros sabéis susurrame en el momento oportuno. Porque no sólo son las palabras, sino vuestra especial manera de transmitirme cada sílaba. A vuestro lado la vida era mucho más fácil e infinitamente más bonita. Sois MI ORGULLO, MI GUÍA, MI MOTIVO PARA NO RENDIRME. Sigo sintiendo vuestro olor, vuestra dulzura y vuestra fuerza. Hoy podéis celebrar que juntos hemos conseguido sacar adelante una Tesis Doctoral. SÓLO VOSOTROS y SÓLO CONMIGO. Porque todo esto es obra vuestra. Ojalá os tuviese aquí, colmándome de felicitaciones y besos de esos que tanto extraño y tanto me gustan. Memé, Pepé, OS QUIERO CON TODA MI ALMA y os llevo en el lugar más confortable de mi corazón. Vivís en mí hoy y siempre. GRACIAS INFINITAMENTE.

AYUDAS Y PROYECTOS DE INVESTIGACIÓN

Esta Tesis Doctoral ha sido realizada gracias a la ayuda/financiación otorgada por:

- **Contrato predoctoral** Xunta de Galicia (Plan I2C) modalidad B en el ámbito del Campus de Excelencia Internacional *Campus do Mar*, de 12/2012 a 12/2015.

- **Axuda a estadías predoutorais INDITEX-UDC 2015,** para la **realización de estancias** de investigación en el **extranjero.** Estancia internacional llevada a cabo en el Instituto da Água da Região Norte (IAREN) en Matosinhos, Porto (Portugal), de septiembre a diciembre de 2015.

El trabajo desarrollado forma parte de los siguientes proyectos de investigación:

Estudio de la Implementación de la Directiva Marco del Agua al Medio Marino en Galicia. (Ref. 09MDS038103PR).

Entidad financiadora: Consellería de Economía e Industria da Xunta de Galicia. Programa Sectorial de Investigación Aplicada de Tecnología del Medio Natural y Desarrollo Sostenible del Plan Galego de Investigación, Desenvolvemento e Innovación Tecnolóxica (INCITE). Xunta de Galicia.

Duración: desde: 2009 hasta: 2011

Investigador responsable: Soledad Muniategui Lorenzo

Programa de Consolidación e Estructuración de Unidades de Investigación Competitivas (Ref. Expte. 52/2010).

Entidad financiadora: Consellería de Educación e Ordenación Universitaria. Dirección Xeral de Promoción Científica e Tecnolóxica do SUG. Xunta de Galicia.

Duración: desde: 2010 hasta: 2012

Investigador responsable: Darío Prada Rodríguez (Coordinador Grupo QANAP).

Entidades participantes: Grupo de Investigación "Química Analítica y Aplicada" (QANAP) (UDC/G000343).

Número de investigadores participantes: 30.

Programa de Consolidación e Estructuración de Unidades de Investigación Competitivas (Ref. GRC 2013-047).

Entidad financiadora: Consellería de Educación e Ordenación Universitaria. Dirección Xeral de Promoción Científica e Tecnolóxica do SUG. Cofinanciación Fondo Europeo de Desarrollo (FEDER).

Duración: desde: 2013 hasta: 2016

Investigador responsable: Darío Prada Rodríguez (Coordinador Grupo QANAP).

Entidades participantes: Grupo QANAP (UDC/G000343).

Impacto de microplásticos, contaminantes regulados y emergentes en ecosistemas marinos y establecimiento de sus criterios de calidad ambiental (IMPACTA) (REF. CTM201348194-C3-2-R).

Entidad financiadora: Programa Estatal de Investigación, Desarrollo e Innovación Orientada a los Retos de la Sociedad. Convocatoria 2013. Ministerio de Economía y Competitividad. Financiación FEDER (50%).

Duración: desde 1/1/2014 hasta 31/12/2016

Investigador responsable: Soledad Muniategui Lorenzo.

Proyecto coordinado: Instituto Español de Oceanografía (IP Coordinador, Víctor Manuel León), Universidade da Coruña (Subproyecto 2) y Universidad de Vigo (IP Ricardo Beiras, Subproyecto 3).

ÍNDICE

ACRÓNIMOS Y ABREVIATURAS

AcEt	Acetato de etilo	Ethyl acetate
ACN	Acetonitrilo	Acetonitrile
ADN	Ácido desoxirribonucleico	Deoxyribonucleic acid
APCI	Ionización química a presión atmosférica	Atmospheric pressure chemical ionization
API	Ionización a presión atmosférica	Atmospheric pressure ionization
APPI	Fotoionización a presión atmosférica	Atmospheric pressure photoionization
ARN	Ácido ribonucleico	Ribonucleic acid
BEH	Puentes híbridos de etileno	Ethylene bridged hybrid
C_8	Octilsilano	Octylsilane
C_{18}	Octadecilsilano	Octadecylsilane
CI	Ionización química	Chemical ionization
CMA	Concentración máxima admisible	Maximum permissible concentration
C-ZrO_2	Columna de carbono recubierta de zirconio	Carbon column Zirconium coated
DAD	Detector de red de diodos	Diode array detector
DCM	Diclorometano	Dichloromethane
DEA	Desetil-Atrazina	Desethyl-Atrazine
DEDIA	Desetil-desisopropil-Atrazina	Desethyl-desisopropyl-Atrazine
DEHA	Desetil-2-hidroxi-Atrazina	Desethyl-2-hidroxy-Atrazine
DET	Desetil-Terbutilazina	Desethyl-Terbuthylazine
DIA	Desisopropil-Atrazina	Desisopropyl-Atrazine
DIHA	Desisopropil-2-hidroxi-Atrazina	Desisopropyl-2-hidroxy-Atrazine
DI-SDME	Microextracción en gota por inmersión directa	Direct inmersión single drop
DLLME	Microextracción líquido-líquido dispersiva	Dispersive liquid-liquid microextraction
DMSPE	Microextracción en fase sólida dispersiva	Dispersive solid phase microextraction

DNOC	Dinitro-ortocresol	Dinitro-orthocresol
dSPE	Extracción en fase sólida dispersiva	Dispersive solid phase extraction
ECD	Detección por captura de electrones	Electron-capture detection
EDCs	Compuestos disruptores endocrinos	Endocrine disrupting compounds
EFSA	Autoridad Europea de Seguridad Alimentaria	European Food and Safety Authority
EPA	Agencia para la Protección del Medio Ambiente	Agency for Environmental Protection
ESI	Ionización por electrospray	Electrospray ionization
EQS	Normas de calidad ambiental	Environmental Quality Standards
FAB	Ionización por bombardeo con átomos rápidos	Ionization by fast atom bombardment
FAO	Organización para la Alimentación y la Agricultura	Food and Agriculture Organization
FDA	Administración para drogas y alimentos	Food and Drug Administration
GC	Cromatografía de gases	Gas chromatography
GCB	Carbón negro grafitizado	Graphitized carbon black
GPC	Cromatografía de gel permeación	Gel permeation chromatography
HA	2-hidroxi-Atrazina	2-hidroxy-Atrazine
HF-LLME	Microextracción líquido-líquido en fibra hueca	Hollow fiber liquid-liquid microextraction
HF-LPME	Microextracción en fase líquida con fibra hueca	Hollow fiber liquid phase microextraction
HPLC	Cromatografía de líquidos de alta resolución	High performance liquid chromatography
HS	2-hidroxi-Simazina	2-hidroxy-Simazine
HT	2-hidroxi-Terbutilazina	2-hidroxy-Terbuthylazine
IS	Patrón interno	Internal standard
IT	Trampa iónica cuadrupolar	Quadrupolar ion trap

$K_{o/w}$	Coeficiente de reparto octanol/agua	Octanol/water partition coefficient
LC	Cromatografía de líquidos	Liquid chromatography
LIT	Trampa iónica lineal	Linear ion trap
LLE	Extracción líquido-líquido	Liquid-liquid extraction
LLME	Microextracción líquido-líquido	Liquid-liquid microextraction
LLSME	Microextracción sólido-líquido-líquido	Solid-liquid-liquid microextraction
LMRs	Límites máximos de residuos	Maximum residue limits
LOD	Límite de detección	Limit of detection
LOQ	Límite de cuantificación	Limit of quantification
LPME	Microextracción en fase líquida	Liquid phase microextraction
m/z	Relación masa/carga	mass/charge ratio
MAE	Extracción asistida por microondas	Microwave assisted extraction
MALDI	Desorción/Ionización de matriz asistida por láser	Matrix assisted laser desorption/ionization
MARM	Ministerio de Medio Ambiente y Medio Rural y Marino	Ministery of Environment and and Rural and Marine Medium
MCPA	2-metil-4-clorofenoxiacético	2-methyl-4-chlorophenoxyacetic
MeOH	Metanol	Methanol
MIPs	Polímero de impresión molecular	Molecular imprinted polymer
MRM	Monitorización de reacciones múltiples	Multiple reaction monitoring
MS	Espectrometría de masas	Mass spectrometry
MS/MS	Espectrometría de masas en tándem	Tandem mass spectrometry
MSFD	Directiva Marco sobre la Estrategia Marina	Marine Strategy Water Framework
MSPD	Dispersión de la matriz en fase sólida	Matrix solid phase dispersion
MTBE	Metil-terbutil-éter	Methyl-terbuthyl-ether
NCA	Normas de calidad ambiental	Environmental Quality Standards
NHR	Amina	Amine
OCPs	Pesticidas organoclorados	Organochlorine pesticides

OMS	Organización Mundial de la Salud	World Health Organization
PAHs	Hidrocarburos policíclicos aromáticos	Polycyclic aromatic hydrocarbons
PCBs	Policlorobifenilos	Polychlorinated biphenyls
PCX	Material polimérico de Intercambio	Polymeric cation exchange material
PDBEs	Bifenilos polibromados	Polybrominated biphenyls
PDMS	Polidimetilsiloxano	Polydimethylsiloxane
PLE	Extracción con líquidos a presión	Pressurized liquid extraction
PSA	Fase polimérica enlazada de etilendiamina-N-propil	Polymeric bonded phase ethylenediamine-N-propyl
PTFE	Politetrafluoroetileno	Polytetrafluoroethylene
PVP-DVB	Poli (N-vinilpirrolidina-divinilbenceno)	Poly (N-vinylpyrrolidine-divinylbencene)
q	Celda de colisión	Collision cell
Q	Cuadrupolo	Quadrupole
QqQ	Triple cuadrupolo	Triple quadrupole
QuECHeRS	Rápido, fácil, barato, efectivo, robusto y seguro	Quick, Easy, Cheap, Effective, Rugged and Safe
RF	Radiofrecuencia	Radiofrequency
R	Recuperación	Recovery
RD	Real Decreto	Royal decree
RSD	Desviación estándar relativa	Relative standard deviation
SAX	Intercambiador aniónico fuerte	Strong anion exchanger
SBSE	Extracción con barras agitadoras	Stir bar sorptive extraction
SCX	Intercambiador catiónico fuerte	Strong cation exchanger
SDME	Microextracción en gota	Single drop microextraction
SDS	Dodecilsulfato de sodio	Sodyum dodecyl sulphate
SIM	Monitorización de iones selectivos	Selected ion monitoring
SIMS	Espectrometría de masas de	Secondary ion mass spectrometry

SPE	Extracción en fase sólida	Solid phase extraction
SPME	Microextracción en fase sólida	Solid phase microextraction
SRM	Monitoreo de reacción selectiva	Selected reaction monitoring
$t_{1/2}$	Tiempo de vida media	Average life time
TD	Desorción térmica	Thermal desorption
TOF	Tiempo de vuelo	Time of flight
TS	Termonebulización	Thermospray
UPLC	Cromatografia de líquidos de ultra-resolución	Ultra performance liquid chromatography
UICN	Unión Internacional para la Conservación de la Naturaleza	International Union for Nature Conservation
US	Ultrasonidos	Ultrasonic
UV	Ultravioleta	Ultraviolet
UV/Vis	Ultravioleta/Visible	Ultraviolet/visible
VALLME	Microextracción líquido-líquido asistida por agitación vórtex	Vortex-assisted liquid-liquid microextraction
WFD	Directiva Marco del Agua	Water Framework Directive

RESUMEN/RESUMO/ABSTRACT

Las triazinas son uno de los grupos de herbicidas más utilizados para eliminar las malas hierbas. Actualmente están considerados medioambientalmente peligrosos debido a su persistencia, toxicidad, capacidad de bioacumulación y por ser disruptores endocrinos. Su empleo indiscriminado se manifiesta en suelos, frutas y verduras, pero también en el agua cuando estos contaminantes son arrastrados por las lluvias y los arroyos. Después de su aplicación una gran proporción permanece en el medio ambiente y, debido a su persistencia y movilidad, alcanzan el medio marino.

A pesar de que para la mayoría de las triazinas no se han regulado límites máximos en aguas, sedimentos o biota, la *Directiva 2013/39/UE* advierte sobre la importancia de monitorizar contaminantes emergentes que, aunque no están considerados en los programas de control, pueden tener efectos tóxicos. La Unión Europea ha incluido Simazina y Atrazina en la lista de las 33 sustancias prioritarias en la *Directiva Marco del Agua (2000/60/CE)*, por medio de la *Decisión 2455/2001/CE*. Además, la *Directiva 2008/105/CE* establece las normas de calidad ambiental (NCA) para estos compuestos en las aguas superficiales, e insta a los Estados Miembros a establecer parámetros de calidad para estos compuestos en sedimentos y biota a nivel nacional. Por último, la *Directiva 2013/39/UE* incluye la Terbutrina en la lista de sustancias prioritarias.

Por otra parte, la *Directiva Marco de Estrategia Marina 2008/56/CE* establece entre los descriptores de buena salud ambiental que "los contaminantes presentes en el pescado y otros productos de la pesca destinados al consumo humano no superen los niveles establecidos por la normativa comunitaria". Por otro lado, el *Reglamento 396/2005/CE*, relativo a los límites máximos de residuos de plaguicidas en alimentos y piensos de origen vegetal y animal, establece límites para Simazina y Terbutilazina en algas, aunque todavía no ha establecido límites máximos para pesticidas en animales acuáticos. A este respecto, la Agencia Americana *Food and Drug Administration* sí ha establecido niveles de tolerancia para pesticidas, entre los que se encuentra la Simazina, en pescados y productos de la pesca.

Los herbicidas triazínicos pueden ser transformados por procesos químicos y biológicos. Debido a su movilidad, los productos de degradación pueden llegar a los cuerpos de agua más fácilmente que las triazinas; por lo tanto, el impacto debido a los herbicidas tiende a subestimarse cuando sólo las triazinas son analizadas, siendo por ello necesario incluir sus principales productos de degradación para obtener un mejor conocimiento en relación con la contaminación por herbicidas.

Por todo esto, es necesario disponer de métodos de análisis precisos, eficaces, sencillos y rápidos para estos compuestos. Así, a lo largo de esta Tesis Doctoral, se ha desarrollado metodología analítica para la determinación de estos compuestos a niveles

traza tanto en agua de mar como en sedimento y biota. Estos métodos mejoran los propuestos en la bibliografía, son sensibles, selectivos y simples, cumpliendo además con los principios de la Química Verde. Una vez validados los métodos de análisis, se realizó un muestreo de aguas de mar, sedimentos y biota acuática (algas, peces y moluscos).

As triazinas constitúen un dos grupos de herbicidas máis amplamente utilizados para eliminar as malas herbas. Actualmente son considerados medioambientalmente perigosos por mor da súa persistencia, toxicidade, capacidade de bioacumulación e por seren disruptores endócrinos. O seu emprego indiscriminado ponse de manifesto nos solos, froitas e verduras, pero tamén nas augas, cando estes contaminantes son arrastrados polas choivas e os regatos. Tras a súa aplicación, unha grande proporción permanece no medio ambiente e, debido á súa persistencia e mobilidade, acadan o medio mariño.

A pesar de que para a meirande parte das triazinas non se teñen regulado límites máximos nas augas, sedimentos ou biota, a *Directiva 2013/39/UE* advirte sobre a importancia de monitorizar contaminantes emerxentes que aínda que non estean considerados nos programas de control, poidan ter efectos tóxicos. A Unión Europea incluiu Simazina e Atrazina no listado das 33 sustancias prioritarias na *Directiva Marco da Auga (2000/60/CE)*, mediante a *Decisión 2455/2001/CE*. Ademais, a *Directiva 2008/105/CE* estabelece as normas de calidade ambiental (NCA) para estes compostos nas augas superficiais, e insta aos Estados Membros a estabelecer parámetros de calidade para estes compostos en sedimentos e biota a nivel nacional. Por último, a *Directiva 2013/39/UE* inclúe a Terbutrina no listado de sustancias prioritarias.

Por outra banda, a *Directiva Marco de Estratexia Mariña 2008/56/CE* estabelece entre os descritores de boa saúde ambiental que "os contaminantes presentes nos peixes e produtos da pesca destinados ao consumo humano non superen os niveis estabelecidos pola normativa comunitaria". Neste senso, o *Regulamento 396/2005/CE*, relativo aos límites máximos de residuos de praguicidas en alimentos e pensos de orixe vexetal e animal, estabelece límites para Simazina e Terbutilazina en algas, emporiso aínda non ten estabelecido límites máximos para pesticidas en animais acuáticos. A este respecto, a Axencia Americana *Food and Drug Administration* ten estabelecido niveis de tolerancia para pesticidas, entre os que se atopa a Simazina, en peixes e produtos da pesca.

Os herbicidas triazínicos poden ser transformados por procesos químicos e biolóxicos. Debido á súa mobilidade, os produtos de degradación poden chegar aos corpos de auga máis doadamente cás triazinas; como consecuencia, o impacto debido aos herbicidas tende a subestimarse cando só as triazinas son analizadas, resultando necesario incluír os seus principais produtos de degradación para obter un mellor coñecemento en relación á contaminación por herbicidas.

Por todo o exposto, cómpre dispoñer de métodos de análise precisos, eficaces, sinxelos e rápidos para estes compostos. Así, ao longo desta Tese Doutoral, desenvolveuse metodoloxía analítica para a determinación destes compostos a niveis

traza tanto en auga de mar como en sedimento e biota. Estes métodos melloran os propostos na bibliografía, son sensíbeis, selectivos e sinxelos, cumprindo ademais cos principios da Química Verde. Unha vez validados os métodos de análise, realizouse unha mostraxe de augas de mar, sedimentos e biota acuática (algas, peixes e moluscos).

Triazines are one of herbicides groups most widely used for the control of weeds. Currently, they are considered environmentally dangerous because of their persistence, toxicity, bioaccumulation and being endocrine disruptors. Their indiscriminate use can be observed not only in soils, fruits and vegetables, but also in water when these pollutants are washed away by rain. After their application, a large amount remains in the environment and, because of their persistence and mobility, can reach the marine environment.

Although there are no maximum limits regulated for most of the triazines in water, sediment or biota, the *Directive 2013/39/EU* calls the attention on the importance of monitoring emerging pollutants which are not considered in the control programs, but can have toxic effects. The European Union has included Simazine and Atrazine in the list of 33 priority substances in the *Water Framework Directive (2000/60/EC)*, by way of *Decision 2455/2001/EC*. Moreover, the *Directive 2008/105/EC* sets the Environmental Quality Standards (EQS) for these compounds in surface waters and committees the Member States to set EQS for these compounds in sediments and biota at national level. Finally, the *Directive 2013/39/EU* adds Terbutryn to the list of priority substances.

On the other hand, the *Marine Strategy Framework Directive 2008/56/EC* establishes, between descriptors of good environmental health, "contaminants in fish and other fishery products destined to human consumption should not exceed the levels established by Community legislation". In this way, the *Regulation 396/2005/EC*, relative to the maximum residue levels of pesticides in food and feed of plant and animal origin, sets limits for Simazine and Terbuthylazine in algae; however this Regulation has not established yet maximum levels for pesticides in aquatic animals. Regarding to it, the *U.S Food and Drug Administration* has set tolerance levels for pesticides, including Simazine, in fish and fishery products.

Triazinic herbicides may be transformed by chemical and biological processes. Because of their mobility, the degradation products can reach water bodies more easily than triazines; therefore, the impact due to herbicides tends to be underestimated when only the triazines are analyzed, being necessary to include their main degradation products to obtain a better knowledge regarding herbicides pollution.

Therefore, it is necessary to provide precise, effective, simple and quick analytical methods for these compounds. Thus, throughout this Thesis, analytical methodology for the determination of these compounds in seawater, sediment and biota at trace levels was developed. The proposed methods have improved the previous methodologies found in the literature, are sensitive, selective and simple, besides

complying with Green Chemistry principles. Once analytical methods were validated, a sampling of seawater, sediment and aquatic biota (algae, fish, and mollusks) was held.

OBJETIVOS

Esta Tesis Doctoral se engloba en una de las líneas de investigación del grupo de Química Analítica Aplicada (QANAP) de la Universidade da Coruña, centrada en el desarrollo de metodologías analíticas en los campos ambiental, industrial y agroalimentario.

La contaminación química de las aguas superficiales representa una amenaza para el medio marino con efectos de toxicidad aguda y crónica para los organismos acuáticos, acumulación en el ecosistema y pérdidas de hábitats y de biodiversidad, con repercusión en la salud humana. Entre estas sustancias químicas contaminantes se encuentran los herbicidas triazínicos y sus productos de degradación; un ejemplo es la Atrazina que produce daños genotóxicos en los peces. La evaluación del ecosistema marino implica tanto la estimación de la calidad del agua como de los sedimentos y los organismos biológicos. Por todo ello, es necesario desarrollar y validar métodos de análisis sensibles y selectivos para la determinación de las triazinas y sus principales productos de degradación en agua de mar, sedimento y biota (algas, pescados y mariscos) que permitan alcanzar los límites establecidos en la legislación.

En esta Tesis Doctoral se pretende proveer a la comunidad científica y a los laboratorios de rutina de una metodología analítica que permita la monitorización de nueve triazinas (Ametrina, Atrazina, Cianazina, Prometrina, Propazina, Simetrina, Simazina, Terbutilazina y Terbutrina) seleccionadas por ser las más empleadas en la formulación de mezclas de herbicidas triazínicos, estando algunas de ellas legisladas, y sus ocho principales productos de degradación (Desetil-Atrazina, Desetil-Desisopropil-Atrazina, Desetil-2-Hidroxi-Atrazina, Desetil-Terbutilazina, Desisopropil-Atrazina, Desisopropil-2-Hidroxi-Atrazina, 2-Hidroxi-Atrazina y 2-Hidroxi-Terbutilazina).

Por lo comentado anteriormente, los objetivos generales de esta Tesis Doctoral son los siguientes:

1. Desarrollo y validación de nuevas metodologías analíticas para la determinación de triazinas y sus productos de degradación en agua de mar y sedimentos marinos.
2. Desarrollo y validación de nuevas metodologías analíticas para la determinación de herbicidas triazínicos en biota (algas, mejillón y trucha).
3. Estudio de los niveles de estos contaminantes en el medio marino en diferentes áreas de Galicia y Portugal, así como en biota acuática destinada al consumo humano.
4. Diseño de una actividad docente, relacionada con la investigación desarrollada, para los alumnos del Grado en Química.

CAPÍTULO I

INTRODUCCIÓN

1. HERBICIDAS. TRIAZINAS

El rápido crecimiento que la producción agrícola ha venido experimentando en las últimas décadas se debe, entre otras causas, a la protección que diversos cultivos han recibido mediante la aplicación de herbicidas que han disminuido las pérdidas de cosechas ocasionadas por las malas hierbas. Sin embargo, el amplio uso de herbicidas en viñedos, huertos, jardinería y diferentes cultivos tiene consecuencias negativas sobre el medioambiente, donde se dispersan y se degradan contaminando las aguas, la atmósfera, el suelo y los alimentos, llegando por último a los seres vivos *(Cobb y Kirkwood, 2000; LeBaron et al., 2008).*

Los primeros herbicidas fueron sales inorgánicas, tales como sulfato de cobre, pero el primer herbicida orgánico, dinitro-ortocresol (DNOC), no fue introducido hasta 1932. El uso extensivo de herbicidas comenzó en 1945 con el lanzamiento de los herbicidas reguladores de crecimiento: ácido 2,4-diclorofenoxiacético (2,4-D) y ácido 2-metil-4-clorofenoxiacético (MCPA). El éxito de éstos condujo a una intensificación de la investigación, produciéndose nuevos grupos de herbicidas. En la actualidad siguen siendo una herramienta crucial para la producción agrícola mundial *(Abbas et al., 2015),* a pesar de estar considerados medioambientalmente peligrosos por su toxicidad, por su capacidad de bioacumulación y por ser disruptores endocrinos *(LeBaron et al., 2008).*

Así, en las últimas décadas, se han detectado restos de herbicidas (incluidas triazinas) en zonas bastante alejadas de su punto de aplicación, tanto en aguas subterráneas como superficiales e incluso en sedimentos. Por esta razón, el impacto de los agroquímicos tanto en la calidad del agua como en el medio acuático en general, se ha convertido en un asunto de importancia mundial *(Klementova et Keltnerova, 2015).*

La penetración de los herbicidas en las plantas se efectúa en condiciones muy diferentes según se produzca por vía aérea o por vía subterránea. En el caso de la penetración foliar, el herbicida está muy concentrado y a menudo es absorbido en menos de 24 horas, mientras que en el suelo se encuentran en menor concentración siendo adsorbidos durante un largo periodo de tiempo *(De Prado et Jorrín, 2001).*

Los herbicidas se pueden clasificar, atendiendo a su estructura química, en 18 familias: herbicidas inorgánicos, ácidos haloalcanoicos, ácidos fenoxialcanoicos, ácidos aromáticos, amidas, nitrilos, anilidas, nitrofenoles, nitrofeniléteres, nitroanilidas, carbamatos, tiocarbamatos, derivados de la urea, compuestos nitrogenados heterocíclicos, compuestos organoarsenicales, organofosforados, sulfonilureas e imidazolinonas *(LeBaron et al., 2008).* En el presente trabajo los herbicidas objeto de estudio son las triazinas, que son compuestos nitrogenados heterocíclicos.

1.1. Estructura química

Las triazinas presentan una estructura heterocíclica, análoga a la del benceno, en la que tres átomos de nitrógeno sustituyen a tres átomos de carbono. La introducción de tres átomos de nitrógeno en el núcleo bencénico genera tres isómeros estructurales: 1,3,5-Triazina (simétrica); 1,2,4-Triazina (asimétrica) y 1,2,3-Triazina (vecinal), cuyas estructuras se muestran en la **figura 1.1.**

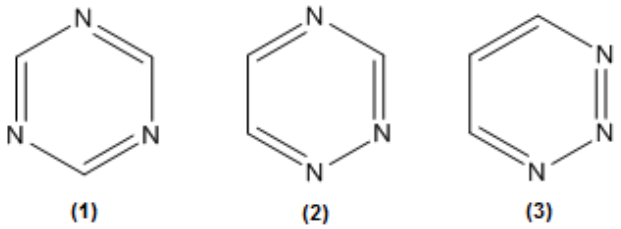

Figura 1.1. Estructura química de las Triazinas (1) isómero 1,3,5-Triazina, (2) isómero 1,2,4-Triazina y (3) isómero 1,2,3-Triazina

El más importante de estos isómeros es la 1,3,5-Triazina, llamada también s-triazina, que por tener baja solubilidad en agua probablemente tenga una menor distribución en el medio ambiente que los otros isómeros de mayor solubilidad, y por ello es la que presenta un mayor interés. Una gran parte de los herbicidas triazínicos derivan de dicho isómero. Los derivados de la s-triazina se forman por la adición de sustituyentes en las posiciones 2, 4 y 6. En la posición 2 se suele introducir un grupo funcional metoxi (-OCH_3), tioalquil (-SCH_3) o cloruro (-Cl), mientras que en las posiciones 4 y 6 se introducen grupos amino sustituidos (-NHR) tal y como se recoge en la **figura 1.2.**

El nombre químico del pesticida depende del sustituyente situado en la posición 2. Cuando el sustituyente es un grupo –Cl, se denominan Clorotriazinas y la terminación del nombre lleva sufijo –azina, mientras que si el sustituyente es un grupo –SCH_3 se denominan Metiltiotriazinas y el nombre de la triazina finaliza con el sufijo –trina. En el caso del grupo –OCH_3, se denominan Metoxitriazinas y el sufijo es –ton *(Abbas et al., 2015).*

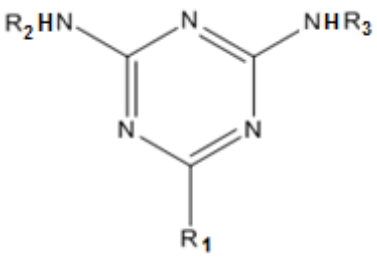

Figura 1.2. Estructura química de las s-triazinas

En este trabajo se estudian cinco Clorotriazinas: 6-cloro-N^2-etil-N^4-isopropil-1,3,5-triazina-2,4-diamina (Atrazina), 2-[4-cloro-6-etilamin-(1,3,5) triazina-2- ilamin]-2-metilpropionitrilo (Cianazina), 6-cloro-N^2,N^4-diisopropil-1,3,5-triazina-2,4-diamina (Propazina), 6-cloro-N^2,N^4-dietil-1,3,5-triazina-2,4-diamina (Simazina) y N^2-tert-butil-6-cloro-N^4-etil-1,3,5-triazina-2,4-diamina (Terbutilazina) y cuatro Metiltiotriazinas: N^2-etil-N^4-isopropil-6-metiltio-1,3,5-triazina-2,4-diamina (Ametrina), N,N'-diisopropil-6-metiltio-1,3,5-triazina-2,4-diamina (Prometrina), N^2, N^4-dietil-6-metiltio-1,3,5-triazina-2,4-diamina (Simetrina) y N^2-tert-butil- N^4-etil-6-metiltio-1,3,5-triazina-2,4-diamina (Terbutrina). *(www.chemspider.com).* Sus estructuras aparecen recogidas en la **figura 1.3.**

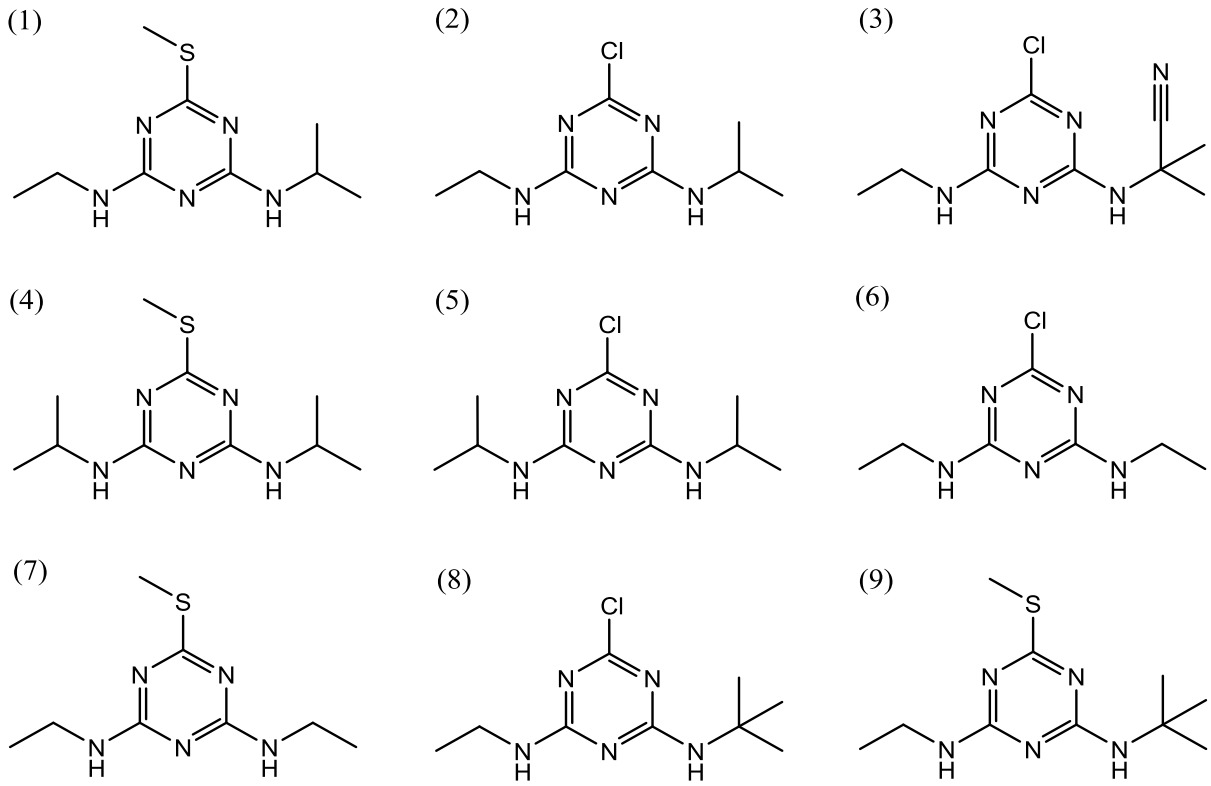

Figura 1.3. Estructuras de las triazinas estudiadas: (1) Ametrina, (2) Atrazina, (3) Cianazina, (4) Prometrina, (5) Propazina, (6) Simazina, (7) Simetrina, (8) Terbutilazina, (9) Terbutrina

1.2. Propiedades físico-químicas

Las propiedades físico-químicas de los herbicidas son las que determinan su movilidad en el medio ambiente. Las propiedades físico-químicas de las *s*-triazinas se deben fundamentalmente a sus sustituyentes, mientras que el anillo sólo tiene efecto en la distribución de cargas. El sustituyente con mayor influencia es el que se encuentra en la posición 2, siendo las propiedades de los cloro derivados bastante diferentes de las de

los metoxi y tiometil derivados. Los grupos aminoalquílicos que se encuentran en las posiciones 4 y 6 no tienen un efecto tan marcado *(LeBaron et al., 2008).*

La acidez se encuentra afectada especialmente por el sustituyente en posición 2, aumentando según el orden $-OCH_3 < -SCH_3 < -Cl$. Los sustituyentes que se encuentran en las posiciones 4 y 6 tienen una cierta influencia; así, en las s-triazinas que sólo difieren en el sustituyente en posición 6, el carácter básico aumenta con la ramificación del grupo aminoalquílico *(LeBaron et al., 2008).*

La solubilidad en agua, aumenta al aumentar la acidez del medio debido a la protonación y depende del sustituyente de la posición 2. La solubilidad de las triazinas aumenta en el orden $-SCH_3 < -Cl < -OCH_3$ *(LeBaron et al., 2008).*

La polaridad se relaciona con el coeficiente de reparto octanol/agua ($\log K_{ow}$) y en las s-triazinas se incrementa de acuerdo con los sustituyentes en posición 2 en el siguiento orden: $-SCH_3 < -Cl < -OCH_3$. Los sustituyentes de las posiciones 4 y 6 disminuyen la polaridad de la molécula al aumentar la longitud de la cadena alquílica *(LeBaron et al, 2008; Abbas et al., 2015).*

Respecto a la absorción de la luz, la mayoría de las triazinas muestran absorción máxima en disolución acuosa en torno a los 220-225 nm cuya intensidad se ve afectada por la acidez de la disolución. En metanol, en el cual las triazinas son mucho más solubles, la longitud de onda máxima aparece a 219-230 nm.

1.3. Fuentes de las triazinas en el medio ambiente

El descubrimiento de las triazinas y su desarrollo tuvo lugar entre 1956 y 1970. Durante ese periodo, se creó una nueva familia de herbicidas que supuso una modernización de la agricultura consiguiendo un éxito en el control de las malezas en los campos de maíz y otros cultivos.

El primer herbicida registrado fue la Simazina, aprobada en Suiza en 1956, utilizándose para eliminar las malas hierbas de las vías del tren y de caminos de paso, y en los campos de maíz, cereales y uvas. El transcurso de los años dio lugar a la aparición de otras triazinas; así en el año 1990, Atrazina, Cianazina, Prometrina y Simazina eran comúnmente empleadas en las tierras de cultivo, mientras que la Propazina había sido eliminada del mercado. La oposición de los agricultores a su retirada del comercio dio lugar a que volviera a registrarse en 2007 *(LeBaron et al., 2008).*

Las triazinas se aplican a gran variedad de cultivos, así como en las vías del ferrocarril, en caminos y en campos de golf. Debido a su baja toxicidad, comparada con la de otros plaguicidas, se han aplicado a cultivos muy extensos tales como campos de

maíz y han sido empleadas en el tratamiento contra plagas que atacan a plantas de raíces profundas, como el olivo, los cítricos, el café, el té, el cacao, etc. *(Huff et Foster, 2011).*

Las triazinas se emplean habitualmente en tratamientos de pre-emergencia y de post-emergencia. Normalmente se aplican a suelos y se absorben a través de las hojas y raíces de las plantas, inhibiendo la fotosíntesis por supresión del transporte de electrones en el fotosistema II, lo que conlleva al impedimento de la absorción de CO_2 y como consecuencia, la muerte de la planta *(LeBaron et al., 2008).*

Por otra parte, los estudios sobre la presencia de algunas triazinas, como Atrazina y Simazina, muestran que su concentración es mayor en agua superficial que en aguas subterráneas. También se han encontrado diferencias según la época del año en la cual se realice el estudio, alcanzando su concentración máxima en primavera y principios de verano, puesto que su aplicación a los campos de cultivos se produce en el mes de abril *(Le Baron et al., 2008).*

1.4 Productos de degradación

Las triazinas tienen una serie de características que las diferencian entre sí, y algunas de ellas tales como la solubilidad, la persistencia o la resistencia que presentan frente a la hidrólisis, hacen que algunos herbicidas triazínicos tiendan a reaccionar y degradarse en mayor grado que otros.

Las consecuencias del uso masivo de pesticidas sigue siendo poco conocido debido a que la mayoría de los estudios se han centrado principalmente en los compuestos padre, por lo que la información sobre el impacto medioambiental originado por sus productos de degradación es limitada. Estos productos pueden ser menos tóxicos que la sustancia original (inactivación), o por el contrario más tóxicos que los productos que las originan (activación) *(Andreu et Picó, 2004).* Debido a su movilidad, estos productos pueden llegar más fácilmente al medio acuático que sus precursores, por lo que el impacto debido a los herbicidas tiende a subestimarse cuando sólo se analizan las triazinas *(Benvenuto et al., 2010).* En estudios recientes sobre herbicidas en aguas superficiales se ha ampliado la lista de compuestos de interés, incluyendo sus principales productos de degradación *(Hildebrant et al., 2008; Huff et Foster, 2011; Köck-Schulmeyer et al., 2012; Bottoni et al., 2013).*

Los pesticidas pueden degradarse mediante procesos químicos, microbiológicos y por fotodescomposición, y en su degradación influyen diferentes factores *(Barchanska et al., 2012).* La degradación química tiene lugar por la acción de enzimas que catalizan determinadas reacciones químicas tales como desalquilación, hidroxilación, fotolisis, hidrólisis, oxidación y reducción.

Esta degradación depende del tipo de suelo, la temperatura, la humedad y de las características del compuesto que va a ser degradado. Las clorotriazinas pierden los átomos de cloro, sufriendo hidroxilación y posterior carboxilación, de modo que se forman compuestos que han perdido su actividad herbicida. Los primeros investigadores que estudiaron esta vía de disipación de las triazinas en el suelo encontraron los metabolitos hidroxilados de Atrazina, Propazina y Simazina *(LeBaron et al., 2008; Krutz et al., 2009).*

Respecto a las reacciones fotoquímicas, que tienen lugar por la absorción de radiación, pueden ser directas o indirectas dependiendo si la energía es absorbida directamente por la triazina o por el medio que la rodea *(Calvet et al., 2005).* La presencia de materia húmica influye en la forma de fotodegradación y acelera su velocidad. La velocidad depende de los sustituyentes que la s-triazina tenga en la posición 2, que aumentan en el orden $-OCH_3 < -Cl < -SCH_3$, y es independiente de la cadena alquílica.

La degradación biológica se debe a la acción de los microorganismos presentes en el suelo, el sedimento y el agua. Esta degradación es lenta y va a verse condicionada por la concentración inicial de herbicida, la cantidad de microorganismos presentes, la humedad, la temperatura o el pH *(Calvet et al., 2005).* Este tipo de degradación se produce fundamentalmente por la N-desalquilación de las cadenas laterales de las triazinas, formando compuestos que aún conservan actividad fitotóxica (*Andreu et Picó, 2004; LeBaron et al., 2008; Krutz et al., 2009).*

Cabe destacar que en las aguas superficiales se produce más fácilmente la degradación que en las aguas subterráneas (*LeBaron et al., 2008*). En este trabajo se estudian los ocho principales productos de degradación. Las estructuras se muestran en la **figura 1.4.**

Cuatro productos de degradación desalquilados: 6-cloro-N-isopropil-1,3,5-triazina-2,4,-diamina (Desetil-Atrazina, DEA), 6-cloro-1,3,5-triazina-2,4-diamina (Desetil-Desisopropil-Atrazina, DEDIA), 2-cloro-4-amino-6-tert-butil-1,3,5-triazina-2,4-diamina (Desetil-Terbutilazina, DET) y 6-cloro-N-etil-1,3,5-triazina-2,4,-diamina (Desisopropil-Atrazina, DIA).

Dos productos de degradación hidroxilados: 2-hidroxi-N^4-etil-N^6-isopropil-1,3,5-triazina-4,6-diamina (2-Hidroxi-Atrazina, HA) y N^2-tert-butil-N^4-etil 6-hidroxi-1,3,5-triazina-2,4-diamina (2-Hidroxi-Terbutilazina, HT).

Dos productos resultantes de la combinación de desalquilación e hidroxilación: 2-hidroxi-N-isopropil-1,3,5-triazina-4,6-diamina (Desetil-2-Hidroxi-Atrazina, DEHA) y

2-hidroxi-N-etil-1,3,5-triazina-4,6-diamina (Desisopropil-2-Hidroxi-Atrazina, DIHA) *(www.chemspider.com).*

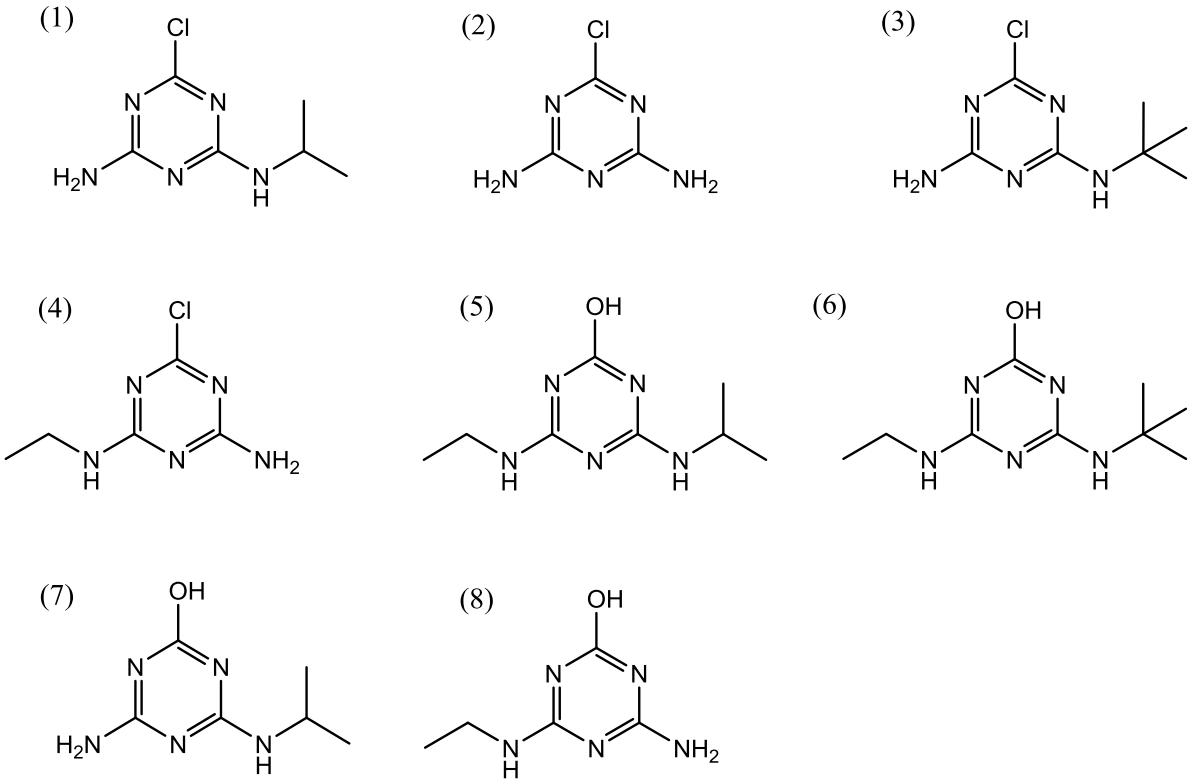

Figura 1.4. (1) DEA, (2) DEDIA, (3) DET, (4) DIA, (5) HA, (6) HT, (7) DEHA, (8) DIHA

Hasta 1993, se creía que los principales productos de degradación de las s-triazinas eran DEA y DIA, formados mediante degradación biológica, es decir, mediante la N-desalquilación de las cadenas laterales. Posteriormente, se demostró que el principal producto de degradación de la Atrazina es HA, que surge principalmente a través de las reacciones biológicas y, en menor medida, de la hidrólisis química siendo DEA y DIA productos minoritarios *(Krutz et al., 2009).*

En el proceso de degradación de la Atrazina en suelos influyen diferentes parámetros como el contenido en materia orgánica, el pH y los microorganismos que estén presentes *(Barchanska et al., 2012),* siendo transformada predominantemente en DEA, DEDIA y DIA mediante la acción de enzimas monooxigenasas. Estos productos de degradación también pueden formarse por la acción de estas enzimas a partir de Propazina y Simazina *(Sabik et al., 2000; Huff et Foster, 2011).*

Las enzimas microbianas necesarias para transformar la Atrazina o sus productos desalquilados en DEHA, DIHA o HA, no están presentes en suelos de manera natural. Sin embargo, el uso repetido de este herbicida ha hecho que mejore la

degradación debido a las bacterias consiguiendo que evolucione la capacidad de transformación de la Atrazina en HA a través de las enzimas Atrazina-clorohidrolasas, especialmente en suelos pobres en nitrógeno y/o carbono *(Huff et Foster, 2011; Barchanska et al., 2012).*

En lo que a aguas se refiere, los productos de degradación más encontrados, producidos por mecanismos bióticos, son los clorometabolitos desalquilados tales como DEA, DET y DIA, mientras que los principales productos de degradación abióticos en aguas son HA y 2-hidroxi-Simazina (HS). Este último no es estable y reacciona dando finalmente un subproducto derivado de la Atrazina y HT *(Benvenuto et al., 2010).* En la **figura 1.5.** se muestran los principales procesos de degradación de la Atrazina.

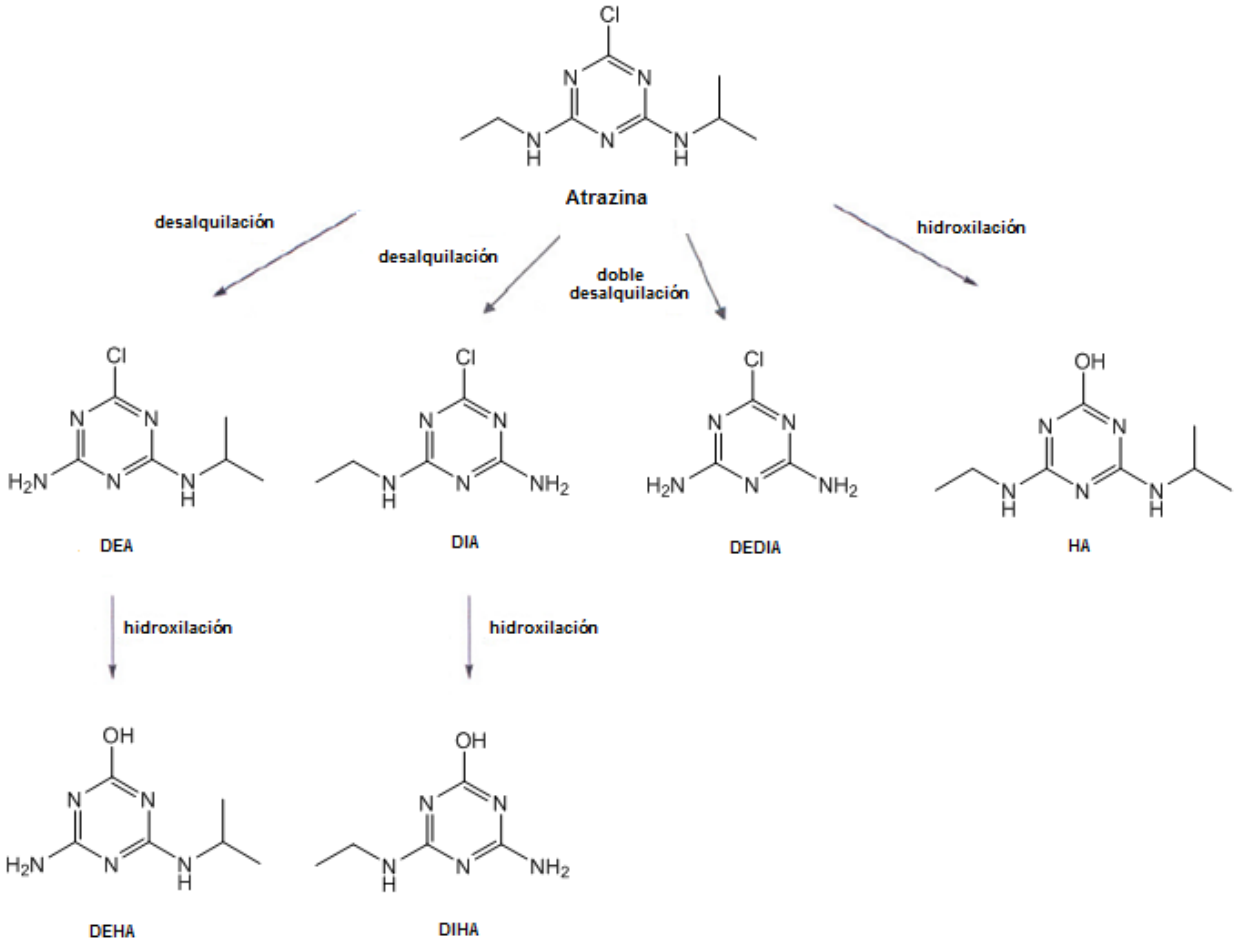

Figura 1.5. Principales procesos de degradación de la Atrazina

Debido a la contaminación ambiental generada por la Atrazina, la Unión Europea prohibió su comercialización y fue reemplazándose de manera gradual por la Terbutilazina, la cual posee menos solubilidad en agua y es más fácilmente adsorbida en suelos *(Papadopoulos et al., 2007; Bottoni et al., 2013).* La actividad herbicida de la

Terbutilazina consiste en inhibir la fotosíntesis debido a la alteración de las proteínas de las membranas de los cloroplastos *(Bottoni et al, 2013).*

Tanto en aguas como en sedimentos, la molécula de Terbutilazina es sometida a diversos procesos de degradación bióticos y abióticos como fotolisis, oxidación, hidrólisis y biodegradación, dando lugar a la desalquilación de los grupos amino, deshalogenación y su posterior hidroxilación *(Papadopoulos et al., 2007; Pinto., 2012).* En el suelo, su degradación se centra en la ruptura de las cadenas laterales e hidrólisis del sustituyente cloro, seguido de mineralización por ruptura del heterociclo, mientras que en el medio acuático se degrada más lentamente porque es estable a hidrólisis y fotólisis.

En la degradación de la Terbutilazina destacan dos reacciones que dan lugar a productos de degradación de elevada persistencia en el medio, la hidroxilación del C_2 de la Terbutilazina transformándola en HT y su desetilación convirtiéndola en DET. Aparte de estas reacciones, pueden tener lugar otros procesos de degradación, mostrándose todos ellos en la **figura 1.6.**

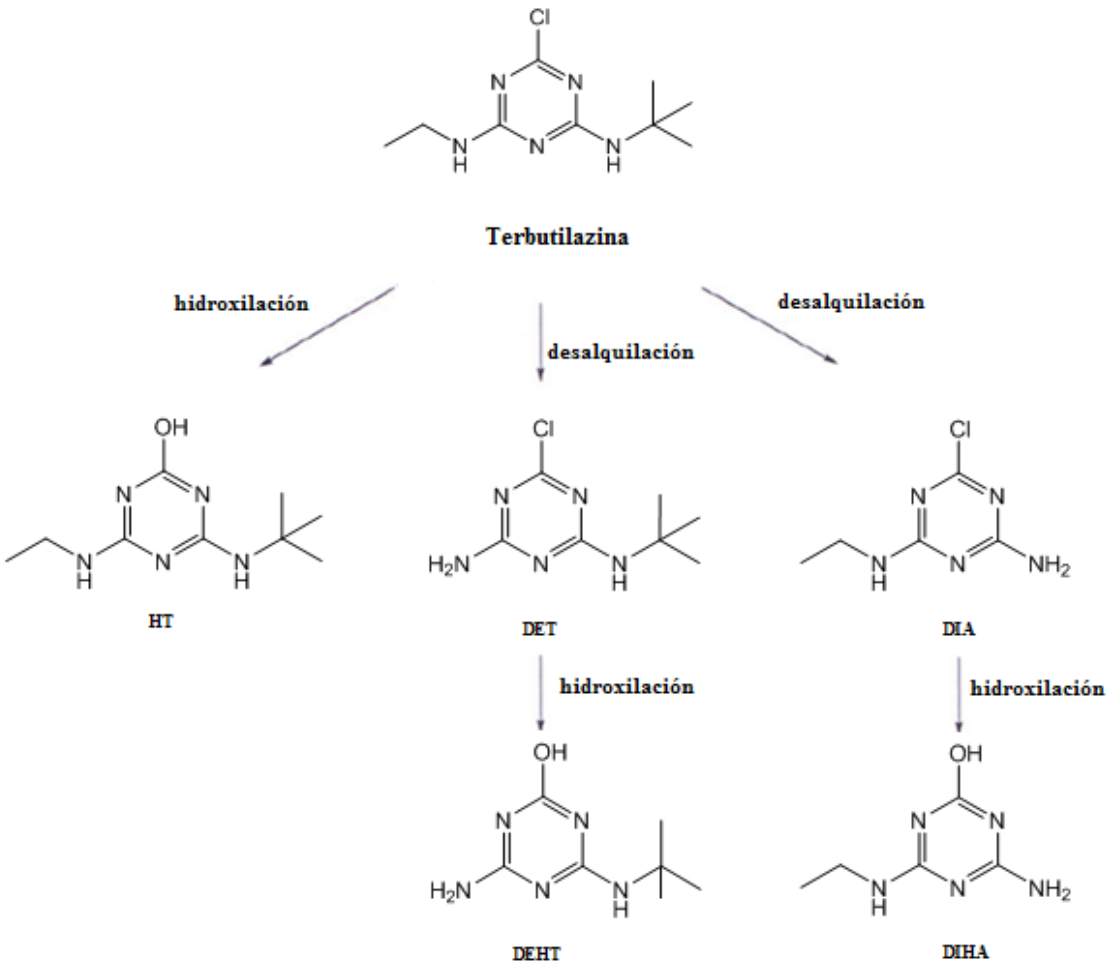

Figura 1.6. Principales procesos de degradación de la Terbutilazina

En lo que respecta a las otras triazinas objeto de este estudio, existe poca información sobre sus productos de degradación. Sin embargo, cabe destacar que la escasa información puede estar relacionada con el hecho de que los metabolitos que se originan a partir de algunas de ellas, como Cianazina, Propazina o Simazina, sean muy inestables dando lugar a productos de degradación derivados de la Atrazina tales como DIA que, aunque se forma directamente a través de la desalquilación de la Atrazina, podría tener más de un precursor. Esto explicaría que en las aguas subterráneas no se hayan encontrado los precursores de este metabolito (Atrazina, Simazina o Terbutilazina), pero se hayan encontrado cantidades elevadas del mismo *(Sabik et al., 2000; Benvenuto et al., 2010).*

1.5 Toxicidad

La toxicidad de las triazinas en los mamíferos es baja comparada con la de otros plaguicidas. Sin embargo, se ha demostrado que pueden tener efectos mutagénicos y patogénicos en organismos vivos, provocando trastornos renales, inhibición de la síntesis de ADN, ARN y proteínas *(LeBaron et al., 2008; LeDoux, 2011).* Con respecto a la biota marina, estudios previos han evidenciado que la exposición crónica a estos compuestos puede causar alteraciones de los sistemas reproductor, hormonal e inmunitario *(Reindl et al., 2015);* también se ha relacionado con trastornos en la actividad neurotransmisora *(Filipov et al., 2007).*

Recientemente se ha descubierto que la Atrazina y otras s-triazinas tales como Cianazina y Simazina, son sospechosas de formar parte de la familia de los disruptores endocrinos (EDCs). Estos compuestos son capaces de alterar el sistema endocrino a muy bajas concentraciones, modificando la función hormonal y causando graves daños *(Olea Serrano et al., 2001).* Presentan distintos mecanismos de actuación y comprenden a un gran número de sustancias con estructuras químicas muy diferentes *(Kalogridi et al., 2014a; Kalogridi et al., 2014b; Abbas et al., 2015).*

Desde los años 30 se conoce la actividad estrogénica de algunos compuestos y se han estudiado en profundidad los compuestos clorados, los cuales formaron parte de la primera lista de Compuestos Orgánicos Persistentes. Sin embargo, a principios de los años 90 comenzó la preocupación por la actividad hormonal de otras sustancias muy diferentes estructuralmente a estos disruptores clásicos. La lista de sustancias que se conoce o sospecha que pueden tener actividad hormonal, entre las que se encuentran las triazinas, supera las 550 y constituyen la lista de sustancias objeto de evaluación de la estrategia comunitaria sobre EDCs *(COM (2001)262 final).* La Comisión Europea considera que está suficientemente probada la capacidad de disrupción endocrina de 118 de estas sustancias y se necesita más información de las restantes.

En relación a la Atrazina, se ha evidenciado que incluso pequeñas cantidades de este compuesto disuelto en agua (0,1 µg/L), puede tener efectos adversos en ciertas especies de anfibios, produciendo hermafroditismo y retraso en desarrollo de las gónadas *(Hayes et al., 2003).* Por otro lado, Moore et al., *(2007)* observaron que la exposición a este compuesto produjo trastornos en la actividad migratoria del salmón del mar Báltico.

Por otra parte, la Autoridad Europea de Seguridad Alimentaria (EFSA) ha informado que la Terbutilazina plantea riesgos elevados a largo plazo para los organismos *(www.efsa.europa.eu)* y puede tener efectos genotóxicos *(Velisek et al., 2016).*

Con respecto a la Ametrina, estudios recientes en ratas de laboratorio indican que presenta efectos genotóxicos, bioquímicos, afectando a la actividad antioxidante de algunas enzimas, y morfológicos, mostrando un descenso significativo en el diámetro y volumen de los hepatocitos *(Santos et al., 2015).*

2. DISTRIBUCIÓN Y PERSISTENCIA EN EL MEDIO AMBIENTE

A pesar de su complejidad, es importante conocer los mecanismos que pueden sufrir las triazinas una vez que entren en contacto con el medioambiente. En muchos casos los herbicidas no permanecen en el lugar en el que se han depositado originalmente, sino que pueden transportarse a grandes distancias a través del agua, del suelo y del aire. Se ha estimado que sólo el 0,1% de los herbicidas aplicados en los cultivos es absorbido, mientras que el resto permanece en el medioambiente *(Li et al., 2010).* Su elevada movilidad y su vida media relativamente alta incrementan su impacto ambiental en distintos hábitats. Dicha movilidad depende no solo de la solubilidad del contaminante en el agua, sino también del coeficiente de reparto octanol/agua y de su contenido en carbono orgánico *(LeBaron et al., 2008).*

La distribución y localización de los herbicidas en el medio ambiente conlleva el estudio de los procesos físicos que controlan el transporte y la difusión de los mismos en el suelo, el aire y el agua *(LeBaron et al., 2008).* Tal y como se puede observar en la **figura 1.7.**, los herbicidas se pueden disipar mediante numerosos procesos entre los cuales cabe destacar la descomposición química, fotoquímica y microbiana, la volatilización, la escorrentía superficial, la lixiviación y la adsorción *(LeBaron et al., 2008).*

El tiempo que una triazina esté inmovilizada en el suelo va a depender de la afinidad existente entre ambos *(Calvet et al., 2005).* Las interacciones dependen del contenido en materia orgánica que tenga el suelo, del pH, humedad y contenido en

arcilla *(LeBaron et al., 2008).* La adsorción disminuye la accesibilidad de los microorganismos a los herbicidas, afectando de este modo a su degradación *(Schrack et al., 2009).*

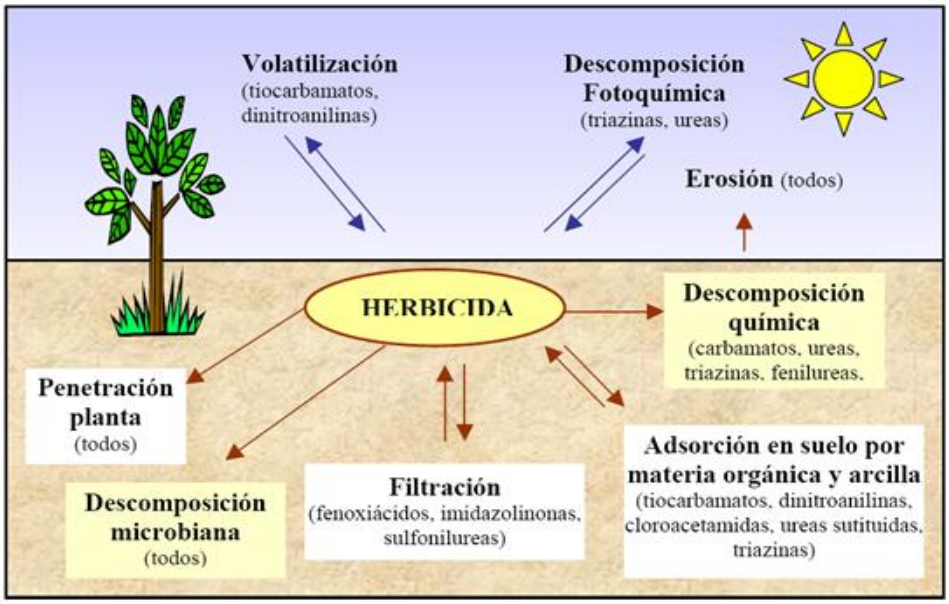

Figura 1.7. Procesos de disipación de los herbicidas en el medio ambiente

Los herbicidas triazínicos no se absorben en su totalidad permaneciendo en el suelo y, debido a sus relativamente bajas presiones de vapor, tienden a evaporarse lentamente pasando de este modo a la atmósfera. Además de la presión de vapor de cada pesticida, hay otros factores que afectan a su volatilización como la temperatura, el contenido en agua del suelo o su adsorción al mismo. La volatilización de los herbicidas y sus productos de degradación constituye un proceso bastante frecuente por lo que es una importante vía de distribución de estos productos al medio ambiente llegando a zonas alejadas del lugar de aplicación *(LeBaron et al., 2008).*

En lo que respecta al medio acuático, el ciclo hidrológico es la principal vía de difusión de los herbicidas desde sus puntos de aplicación a otros compartimentos ambientales, como las aguas subterráneas y superficiales *(Masiá et al., 2013).*

En las aguas subterráneas la lixiviación es el principal proceso de transporte produciéndose por efecto del agua de lluvia o el riego. Ésta se ve afectada por la capacidad de adsorción del suelo, la estructura del mismo y la escorrentía. Con respecto a la adsorción y a la escorrentía, la lixiviación será mayor cuanto menor sean ambos procesos *(Calvet et al., 2005).* En el caso de los suelos de textura áspera que tienen una alta permeabilidad y una gran capacidad de infiltración, el agua de lluvia tiende a infiltrarse en lugar de correr en la superficie *(Kalogridi et al., 2014b).* Otro proceso de

transporte de los herbicidas hacia las aguas subterráneas se produce cuando los pesticidas se asocian a las macromoléculas orgánicas o a las partículas coloidales que están presentes en el suelo *(Calvet et al., 2005).* Por ello, el uso de herbicidas se ha visto limitado en muchos países debido al aumento de compuestos triazínicos observado en las aguas subterráneas *(Abbas et al., 2015).*

La movilización de los herbicidas en las aguas superficiales se produce por escorrentía, ya sea en disolución o adsorbidos por las partículas del suelo desplazándolos hasta distancias considerables *(LeBaron et al., 2008).* La transferencia de los herbicidas a este tipo de aguas se ve influenciada por diferentes factores como el clima, el tipo de suelo o las prácticas de uso de las tierras cultivadas. Si las precipitaciones son muy abundantes y se supera la capacidad de infiltración en el suelo, se va a producir una mayor escorrentía *(Calvet et al., 2005).* La movilización de los herbicidas en las aguas superficiales permite su desplazamiento hasta distancias considerables *(LeBaron et al., 2008).* La **figura 1.8.,** muestra las distintas vías de ingreso de los contaminantes en un medio acuático.

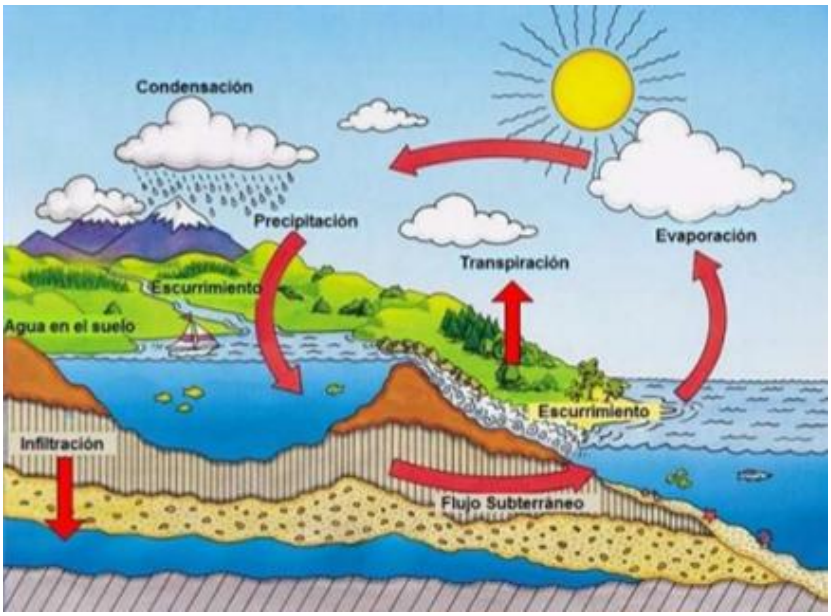

Figura 1.8. Entrada y distribución de los herbicidas en un medio acuático

Una vez que las triazinas y sus productos de degradación llegan al medio acuático, la probabilidad de que estos compuestos alcancen el medio marino es elevada. El medio marino es un ecosistema constituido por elementos bióticos y abióticos interrelacionados entre sí y en equilibrio, por lo que es importante conocer los niveles de residuos contaminantes en todos sus compartimentos, ya que cualquier alteración en alguno de los elementos que lo integran representa un riesgo para los otros. Además, debido a su baja solubilidad y la gran habilidad que poseen las triazinas para asociarse

con partículas, se encuentran presentes en sedimentos y sus efectos acumulativos en el medio costero son considerables *(Camino-Sánchez et al., 2011).*

Algunos herbicidas tienen estructuras químicas muy estables y tardan años en degradarse en formas menos tóxicas. En muchos casos, estos productos son difíciles de eliminar por los organismos vivos por ser poco solubles en agua y tender a acumularse en los tejidos grasos, produciéndose biomagnificación en la cadena trófica. De esta forma, un herbicida que se encuentra en concentraciones muy bajas en el medio acuático, termina estando en concentraciones decenas o cientos de veces más altas en los tejidos grasos de los animales.

El término más corrientemente utilizado para expresar la persistencia de los herbicidas es el denominado "*tiempo de vida media"* ($t_{1/2}$) que se define como "el tiempo necesario para la disipación de la mitad de la cantidad inicialmente presente en el suelo". Este dato puede tener una gran variabilidad debido a la gran cantidad de factores que afectan a la persistencia de un determinado compuesto. En general, los compuestos que tengan vidas medias superiores a 15-20 días deben ser cuidadosamente evaluados por la posibilidad de que contaminen las aguas subterráneas. La persistencia de los herbicidas en el suelo puede variar desde 2 ó 3 semanas hasta más de 6 meses, aunque generalmente se estima en 6 u 8 semanas *(LeBaron et al., 2008).*

El herbicida de las s-triazinas con mayor persistencia es la Terbutilazina *(Carafa et al., 2007)* siendo su vida media en aguas subterráneas de entre 263 y 366 días *(LeBaron et al., 2008).* La Atrazina es altamente persistente en el suelo (más de un año en condiciones secas o frías que no son propicias para la actividad química o biológica) y, debido a su baja adsorción a las partículas del suelo y su larga vida media en aguas (60 a 100 días), presenta un alto potencial de contaminación en aguas subterráneas. En el caso de la Prometrina, aunque es moderadamente persistente en el suelo (de 1 a 3 meses), en condiciones de sequía presenta el mismo comportamiento que la Atrazina y es potencialmente persistente en el medio acuático. La Propazina y la Ametrina también se consideran persistentes en el suelo con una vida media, dependiendo de las condiciones climáticas, entre 35-231 días y 70-250 días respectivamente. Además, la Ametrina debido a su alta solubilidad en agua, puede sufrir lixiviación *(www.epa.gov).*

En lo que respecta a la Simazina, presenta una persistencia moderada en el suelo (8-149 días), y su baja solubilidad en agua limita su potencial de lixiviación. Su vida media en los estanques donde se ha aplicado es de 30 días, aunque depende del nivel de algas, la cantidad de maleza y otros factores *(www.epa.gov).* Por otra parte, Cianazina y Terbutrina tienen baja persistencia en el suelo (2-14 semanas y 14-28 días respectivamente), mientras que en el medio acuático la primera es estable a la acción de la luz solar y a la hidrólisis química y la segunda presenta hidrólisis y degradación lenta

debido a que se adsorbe a los sedimentos *(www.epa.gov)*. Con respecto a la Simetrina, en suelos con bajo contenido en materia orgánica sufre procesos de lixiviación *(www.epa.gov)*, se hidroliza en medios fuertemente ácidos o básicos y se descompone a elevadas temperaturas *(www.pesticidechem.com)*.

3. AGUAS

Las tres cuartas partes de la superficie terrestre están cubiertas de agua formando océanos, mares, ríos, lagos, aguas subterráneas y glaciares. Cabe destacar que el 97% del agua que forma la hidrosfera es agua salada y sólo un 3% es agua dulce. De esta última, el 79% está en forma sólida (hielo y nieve) en los polos y glaciares, el 20% son aguas subterráneas y sólo un 1% del agua dulce forma parte de los ríos y lagos, el suelo, los seres vivos y la atmósfera *(Manahan, 2007)*.

El agua es uno de los elementos más importantes para la vida, no sólo porque es parte integrante de la estructura orgánico-molecular, sino que además participa en innumerables procesos y reacciones químicas, físicas y biológicas. Su capacidad calorífica le permite estabilizar la temperatura de regiones cercanas a los reservorios de agua y su calor de vaporización regula las temperaturas atmosféricas. Es el disolvente en los fluidos biológicos y actúa disolviendo y transportando minerales y nutrientes *(Manahan, 2007)*. Además de ser esencial para el desarrollo de la vida, el agua es un recurso estratégico y económico de primera magnitud en la sociedad actual, sobre todo en lo que se refiere a la dificultad que entraña en muchos casos su obtención y correcta gestión.

El hombre ha incrementado su demanda de agua, tanto debido al crecimiento de la población, como a la agricultura, la industria e incluso para usos lúdicos. Por ello, se está recurriendo al empleo no sólo de las aguas de manantiales y acuíferos, sino también a la utilización de las aguas superficiales *(Rodier, 1990; Miguélez-Pose, 2010; Madrid-Vicente, 2012)*.

La escasez de agua amenaza a más del 40% de la población mundial y se espera que la accesibilidad a fuentes de agua dulce empeore en el futuro, lo cual hace que la desalinización de agua salada se convierta en una necesidad *(Fthenakis et al., 2015)*. Así, en España, las necesidades de agua en algunas zonas del Mediterráneo han llevado a recurrir a plantas de desalinización de aguas salobres y marinas. Estos procesos de desalinización de agua de mar pueden ser fundamentalmente de dos tipos, evaporación y condensación del vapor para obtener un agua dulce de alta calidad o empleo de membranas filtrantes de poros microscópicos obteniendo agua más o menos desalada. Ambos procesos consisten en la separación del agua de mar en un componente de agua

dulce y otro que es un concentrado de salmuera *(Fthenakis et al., 2015; Al-Kalbani et al., 2015).*

Por ello, la polución del medio marino por contaminantes orgánicos como triazinas, está generando una gran preocupación por la elevada distribución de sus residuos en el ecosistema acuático *(Camino-Sánchez et al., 2011; Kalogridi et al., 2014a; Abbas et al., 2015).* Se ha investigado el efecto de las triazinas disueltas en agua en el nivel más bajo de la cadena trófica marina, observándose que la exposición a los derivados de las s-triazinas provoca toxicidad moderada en peces y en sus larvas *(Arufe et al., 2004; Velisek et al., 2016)* y dosis elevadas de estos herbicidas producen cambios patológicos en los órganos internos de los peces *(Wang et al., 2011a).*

Por otra parte, en el medio marino se desarrolla una industria importante que incluye diferentes sectores, no sólo la pesca y el marisqueo, sino también procesos productivos ligados a los cultivos marinos. La acuicultura es una importante actividad y cada vez aumenta más el número de viveros de cultivo en todo el mundo, destacando la cría de moluscos y pescados. Debido a esto, en la última década ha crecido la preocupación sobre la calidad de los productos de acuicultura y la estimación de la calidad del agua en las que se desarrolla el cultivo de las especies, como medida para proteger la salud pública y el medioambiente *(Centro de Cooperación del Mediterráneo de la UICN, 2010).*

Por todo esto, la contaminación química del agua de mar representa una amenaza, no sólo para la salud humana sino también para el medio marino con efectos de toxicidad para los organismos acuáticos, acumulación en el ecosistema, pérdidas de biodiversidad y repercusión sobre los sectores de la industria relacionados con este medio. Este hecho es particularmente importante en Galicia que cuenta con 1700 km de costa, y donde la comercialización tanto de los productos del mar como de sus derivados es una fuente importante de su economía, lo que aumenta la preocupación por el estado de la calidad del agua de sus costas. Por ello, el Centro de Control de Calidad del Medio Marino dependiente de la Consellería de Pesca, Marisqueo y Acuicultura de la Xunta de Galicia realiza un seguimiento de las rías gallegas para conocer y preservar la calidad de las mismas, siendo su principal misión el cumplimiento del Real Decreto 345/1993 por el que se establecen las normas de calidad de las aguas y de la producción de moluscos y otros invertebrados marinos *(Ministerio de Agricultura, Pesca y Alimentación, 2000).*

4. SEDIMENTO

Los sedimentos son partículas procedentes de las rocas o suelos que son acarreadas por las aguas de escorrentía y por el viento. Todos estos materiales

finalmente son depositados a lo largo de los propios cauces, en lagos o lagunas, o en el mar. Los sedimentos naturales están constituidos por una gran variedad de partículas que difieren entre sí en tamaño, forma y densidad. Desde el punto de vista de la resistencia que oponen a ser arrastrados y de su comportamiento al ser transportados por una corriente de agua, se distinguen primordialmente dos clases de sedimentos: cohesivos y no cohesivos *(García-Flores et Maza-Álvarez, 2002).*

El sedimento no cohesivo es el formado por granos gruesos o partículas sueltas, como las arenas y gravas. La fuerza de gravedad predomina fuertemente sobre cualquier otra fuerza; por ello, al disminuir o cesar la acción del agua, las partículas se detienen, caen y se depositan. El sedimento cohesivo es el formado por partículas de grano muy fino, constituidas por minerales de arcilla, que se mantienen unidas entre sí por la fuerza de cohesión, la cual se opone a que las partículas individuales sean separadas. En cuanto a la acción del agua, muchas de ellas (coloides) no se depositan a menos que, por la presencia de sales, se facilite el proceso de floculación.

Los sedimentos son unos componentes muy importantes del entorno marino ya que son el resultado de la integración de todos los procesos (biológicos, físicos y químicos) que ocurren en el ecosistema acuático *(Brondi et al., 2011).* Además constituyen un hábitat para muchos organismos bentónicos y epibentónicos. Por ello influyen en el metabolismo de todo el sistema completo, por lo que pueden proporcionar información muy valiosa sobre la calidad del sistema acuático *(Hela et al., 2005).*

La contribución de los sedimentos a la contaminación química (principalmente fósforo, metales y plaguicidas) está vinculada al tamaño de las partículas de los sedimentos y al contenido en carbono orgánico. Se suele considerar que la fracción químicamente activa de un sedimento es la que mide menos de 63 mm (limo y arcilla). Muchos de los contaminantes persistentes, bioacumulativos y tóxicos, están fuertemente asociados con los sedimentos y en especial con el carbono orgánico transportado como parte de la carga de sedimentos de los ríos. Como consecuencia, los sedimentos pueden actuar a largo plazo como fuente de estas sustancias en el ambiente acuático; de hecho, debido su capacidad para adsorber contaminantes orgánicos, los sedimentos se han propuesto como materiales para reducir la contaminación ambiental de los pesticidas *(Papadopoulos et al., 2012).*

Las propiedades del sedimento que condicionan el movimiento de los pesticidas son la textura, la permeabilidad y su contenido de materia orgánica. A pesar de que en la mayoría de los sedimentos el contenido de materia orgánica es bajo (2-10%), se trata de un componente muy importante de los mismos. La mayor parte del contenido en materia orgánica presente en los sedimentos está formado por sustancias húmicas, que

son macromoléculas ácidas que presentan grupos carboxílicos y fenólicos *(LeBaron et al., 2008).* En lo referente a su textura, ésta afecta a la movilidad del agua a través del sedimento y como consecuencia a la de los productos disueltos en ella, como los herbicidas. La textura de un sedimento depende de las proporciones de arena, arcilla y lodo *(Kalogridi et al., 2014b).* En relación a la permeabilidad, los sedimentos con mayor contenido orgánico retienen más agua. De manera similar, los sedimentos arcillosos y bien estructurados pueden sostener mayor cantidad de agua que los arenosos, mientras que en suelos de textura áspera, como son muy permeables y con elevada capacidad de infiltración, el agua tiende a infiltrarse en el agua subterránea en lugar de ir hacia la superficie.

La retención de los pesticidas en los sedimentos se atribuye principalmente a la arcilla y al contenido de materia orgánica, ya que proporciona numerosos puntos de unión para los pesticidas *(Kalogridi et al., 2014b).* Estos puntos de unión pueden ser polares y apolares. Los puntos de unión polares pueden interaccionar con grupos funcionales presentes en la molécula del herbicida pero también tienen una gran afinidad por el agua, por lo que las moléculas del herbicida van a estar compitiendo con el agua. Los puntos de unión no polares tienen una baja afinidad por el agua, dando lugar a una interacción más fuerte entre el sedimento y los grupos apolares de la molécula del herbicida *(LeBaron et al., 2008).* Generalmente los pesticidas hidrofóbicos, aquellos cuyo log $K_{o/w}$>3, tienen mayor tendencia a enlazarse al sedimento.

Como se dijo anteriormente, la complejidad del ecosistema marino implica que la perturbación de cualquier compartimento repercuta en el resto. Así la presencia de pesticidas en los sedimentos se ha asociado con un amplio rango de impactos en los organismos acuáticos; se han observado episodios de toxicidad aguda y crónica vinculados a pesticidas asociados a partículas de sedimentos en algas, invertebrados, pescados y otros organismos marinos *(Hela et al., 2005; Ricart et al., 2010).*

5. BIOTA

El ecosistema marino nos proporciona una gran cantidad de bienes y servicios que incluyen, entre otros, una fuente vital de alimento para millones de personas. Sin embargo, este ecosistema se encuentra profundamente afectado por causas indirectas como son el cambio climático o las perturbaciones relacionadas con la biogeoquímica del océano, pero también hay causas directas como son la sobreexplotación, la contaminación o la destrucción del hábitat.

En España la tasa de consumo de productos pesqueros es muy alta, siendo líderes de consumo en Europa junto con Noruega y Portugal. Según los datos del Panel

de Consumo elaborado por el Ministerio de Medio Ambiente y Medio Rural y Marino (MARM) *(www.magrama.gob)* en España, aunque tenemos una gran tradición pesquera, actualmente este sector está inmerso en una crisis, por lo que la pesca de captura es muy inferior a la demanda actual, siendo necesario el cultivo de organismos acuáticos (peces, moluscos, crustáceos y plantas acuáticas) *(Padilla-Álvarez, 2009).*

En el siglo XX la acuicultura se ha extendido por todo el mundo, principalmente después de la 2ª Guerra Mundial *(González-Laxe et Fábregas, 2001; Padilla-Álvarez, 2009).* En las últimas décadas esta actividad ha adquirido una gran relevancia tanto por las cifras de producción como por la buena aceptación por los consumidores de los productos procedentes de la acuicultura, incrementándose en consecuencia los niveles de demanda de este tipo de productos *(González-Laxe et Fábregas, 2001; Padilla-Álvarez, 2009; Centro de Cooperación del Mediterráneo de la UICN, 2010).* Recientemente la Unión Europea, mediante la directiva *COM (2012) 494 final*, ha dado un empuje en el crecimiento de la industria de la acuicultura con objeto de satisfacer las demandas de productos pesqueros siendo de esta manera una fuente de empleo y desarrollo económico.

Se pueden diferenciar dos tipos de acuicultura: en medio continental y en medio marino. El factor principal a tener en cuenta para el desarrollo de la acuicultura continental es la disponibilidad y calidad del agua. Respecto al medio marino, las aguas costeras son más ricas que las de los océanos debido a que la escasa profundidad, la gran diversidad de biotopos y el aporte de materiales procedentes del medio terrestre, hacen que el medio cuente con hábitats diversos y que el agua tenga más nutrientes originando una gran densidad de población *(Padilla-Álvarez, 2009).*

Europa es uno de los principales productores acuícolas. Desde 1997 España ocupa la primera posición como productor dentro del sector europeo. De toda la producción comunitaria, aproximadamente el 34,6% se corresponde con la producción de moluscos bivalvos marinos (mejillones en España y ostras en Francia). La producción de peces representa el 65,4 % del total, siendo las principales especies criadas: trucha, salmón, lubina, dorada, anguila y rodaballo. La producción de crustáceos es bastante insignificante (0,01%).

En este sector, Galicia también es uno de los líderes mundiales gracias a sus condiciones naturales, especialmente las rías y a la apuesta realizada desde las empresas y la Administración gallegas. Como consecuencia, la acuicultura gallega ha venido experimentando un continuo crecimiento, tanto en el volumen de su producción como en el valor generado. En el año 2002, la acuicultura gallega alcanzó una producción estimada superior a las 267.000 toneladas, dominando el mercado español y siendo uno de los principales suministradores de los mercados europeos.

De todos los organismos, el mejillón cultivado en las bateas fondeadas en las rías es el producto estrella del sector, ya que goza de un prestigio mundial reforzado por el Consejo Regulador del Mejillón, encargado de velar por la denominación de origen protegida Mejillón de Galicia. Así de las 290.000 toneladas producidas en España en el año 2008, el 78% corresponden a mejillón y, en menor medida a otros moluscos, siendo Galicia origen del 95% de la producción *(Padilla-Álvarez, 2009; Centro de Cooperación del Mediterráneo de la UICN, 2010)*. En lo que respecta a la producción piscícola, de las 64.000 toneladas procedentes de la piscicultura española, 13.000 toneladas, mayoritariamente trucha y rodaballo, se produjeron en Galicia.

Con respecto a las algas marinas, la mayoría de las algas comercializadas crecen sobre las rocas y, una vez recolectadas en la época más adecuada para cada especie, son transportadas a las instalaciones de la empresa para su procesado. Sin embargo, en los últimos años se han venido desarrollando distintos proyectos con el objetivo de instalar cultivos de algas de distintas especies con el fin de satisfacer la creciente demanda y además colaborar en la conservación del medio natural.

El sector de la acuicultura también se enfrenta a una serie de desafíos, que tienen un impacto sobre su viabilidad. El Programa Marino de la Unión Internacional para la Conservación de la Naturaleza (UICN) promueve las mejores prácticas en el sector de la acuicultura con el fin de evitar cualquier posible alteración del medioambiente y para responder a la competencia mundial. Los códigos de conducta y mejores prácticas de acuicultura tienden a centrarse en la reducción del impacto ambiental, la productividad acuícola, la calidad del producto, la salud y bienestar animal, la seguridad alimentaria y los aspectos socioeconómicos *(Centro de Cooperación del Mediterráneo de la UICN, 2010).*

Hoy en día son de gran interés los temas relacionados con la salud pública y los problemas que puede producir la ingesta de alimentos contaminados. En el caso de los organismos acuáticos, éstos son capaces de acumular residuos de pesticidas y otros contaminantes en concentración mayor que el entorno que los rodea *(Ledoux, 2011).* Esta acumulación se produce por difusión a través de las membranas biológicas que se encuentran en contacto directo con aguas contaminadas o por ingestión de alimentos o partículas de sedimentos que contengan estas sustancias. La importancia de cada una de las rutas de entrada varía en función de la situación del organismo considerado en la red trófica acuícola.

Tanto los pesticidas como sus productos de degradación pueden ejercer una acción tóxica en los organismos que habitan en los diferentes compartimentos del sistema acuático. La complejidad del ecosistema acuático y la interdependencia de muchas especies en la cadena trófica provoca que una perturbación en una pequeña

parte del sistema, debida a los efectos tóxicos de los contaminantes, pueda dar lugar a efectos secundarios o indirectos de gran importancia en otro eslabón de la cadena alimentaria *(Hela et al., 2005).*

El interés por la presencia de pesticidas se centra mayoritariamente en organismos capturados o cultivados en áreas expuestas a descargas de contaminantes: aguas continentales, estuarios, zonas costeras así como en los organismos procedentes de la acuicultura *(Food and Drug Administration, 2011).* En el caso de los peces procedentes de acuicultura, los herbicidas también se pueden acumular a través de la alimentación que éstos reciben debido a la ingesta de piensos fabricados con ingredientes contaminados *(Fernández Álvarez et al., 2009)*; además, algunos pesticidas se aplican directamente en los estanques donde se cultiva el pescado para controlar el nacimiento de algas y eliminar organismos invertebrados.

La conservación del medio marino constituye un factor importante para la prosperidad económica y la calidad de vida, y como se ha comentado anteriormente, este hecho es particularmente importante en Galicia. Por ello, el desarrollo de métodos analíticos que permitan determinar los niveles de estos contaminantes en diferentes organismos acuáticos es de gran importancia económica y medioambiental para Galicia.

5.1. Algas

Las algas son un conjunto muy variado de vegetales que viven ligados a la vida acuática. Están formadas por un grupo muy heterogéneo de organismos que comparten la capacidad de realizar la fotosíntesis y cuyo cuerpo vegetativo, llamado talo, carece de verdaderas raíces, tallos y hojas *(Sambamurty, 2006; Llera et Álvarez-Raboso, 2007; Castiñeira, 2011).*

Las algas presentan una amplia distribución geográfica, desde el Ecuador a los Polos, colonizando los medios más diversos, pero siempre ligados a la presencia de agua debido a que no poseen tejidos conductores. Se las puede encontrar viviendo sobre rocas, muros, cortezas de árboles, etc., aunque generalmente son más abundantes en los lagos, ríos y mares poblando la región fótica de los mismos. Las que viven en las aguas pueden ser planctónicas o bentónicas. Las primeras viven en suspensión y son de pequeño tamaño; las bentónicas, se fijan al sustrato y suelen ser de mayor tamaño, incluso de más de 50 metros de longitud *(Sambamurty, 2006; Cabioc´h et al., 2007; Wienckle et Bischof, 2012).*

La diversidad de las algas es extraordinaria y así encontramos infinidad de tipos morfológicos en función de la complejidad de su estructura y adaptaciones especiales de vida. Las menos evolucionadas son unicelulares o coloniales, mientras que las más evolucionadas son las algas pluricelulares que van desde las más sencillas, formadas por

cadenas de células más o menos ramificadas, a las complejas como las laminares, cilíndricas, tubulares, etc. En algunas algas pardas se presenta una cierta complejidad estructural, como sucede en el género Laminaria que por la presencia de unos rizoides, cauloide y filoide, recuerdan a las plantas con verdaderas raíces, tallos y hojas *(Castiñeira, 2011; Wienckle et Bischof, 2012).*

Atendiendo al tamaño podemos encontrar desde especies que no sobrepasan la milésima de milímetro, como sucede en algunas cianofíceas, hasta ejemplares de Laminaria de más de dos metros, e incluso especies como *Nereocystis luetkeana* llegan a sobrepasar los 50 metros *(Llera et Álvarez-Raboso, 2007; Braune et Guiri, 2011).*

La identificación de un macroalga comienza por su color, para así poder determinar a cuál de los cuatro grandes grupos de algas pertenece *(Castiñeira, 2011).*

- *Algas verde-azuladas* (Cyanophyta ó Cianófitos). Son organismos procariontes que contienen pigmentos verde-azulados. Tienen un papel muy importante en la biosfera, pues algunas fijan el nitrógeno atmosférico.
- *Algas rojas* (Rhodophyta ó Rodófitos). Se caracterizan por presentar pigmentos rojos o pardo-rojizos como las fitoeritrinas. Su composición en pigmentos les permite ocupar ambientes muy poco iluminados como zonas profundas y grietas.
- *Algas pardo-doradas* (Phaeophyceae ó Cromófitos). Poseen un pigmento pardo, la flucoxantina y son casi exclusivamente marinas pudiendo alcanzar varios metros de longitud.
- *Algas verdes* (Chlorophyta o Clorófitos). Son de color verde porque la clorofila no queda oculta por otros pigmentos. Pueden colonizar en gran variedad de hábitats.

En las costas atlánticas del norte, las algas rojas son el grupo con mayor número de especies, seguidas de las pardas, que aunque se presentan en menor cantidad son muy aparentes debido al gran tamaño de muchas de ellas, mientras que las algas verdes son las menos frecuentes. En cambio, en el Mediterráneo no existen las grandes algas pardas de nuestras costas y el número de especies de algas rojas y verdes es mucho mayor *(Llera y Álvarez-Raboso, 2007; Braune et Guiri, 2011).*

Las algas son organismos de gran importancia para los ecosistemas marinos, siendo la base de la cadena alimenticia y favoreciendo la oxigenación del agua. Por tanto, el buen estado de los ecosistemas marinos es, en parte, posible gracias al buen estado de las comunidades de algas *(Sambamurty, 2006; Cabioc´h et al., 2007; Wienckle et Bischof, 2012).*

Las comunidades de algas, gracias a su sensibilidad a los cambios del medio en que viven, responden a los impactos antropogénicos convirtiéndose en buenos

indicadores de cambios en la calidad del agua y en un referente del estado ecológico de cualquier sistema acuático. Sus cortos ciclos de vida las convierten en indicadores adecuados para impactos a corto plazo; los hábitos de fijación de la mayoría de las especies hacen que sean afectadas directamente por los cambios físicos y químicos en la columna de agua; por ser productores primarios son sensibles a contaminantes que no tienen efecto sobre organismos heterotróficos, y además son fácilmente muestreables *(Cabioc´h et al., 2007; Wienckle et Bischof, 2012).*

Distintos estudios realizados han mostrado que los cambios en las comunidades algales revelan el inicio de contaminación que pueda existir en un sistema acuático, lo cual se refleja en las modificaciones de la estructura poblacional y en la proliferación de especies asociadas a determinados aportes. Al ser organismos utilizados en sistemas de biomonitorización, se convierten en una herramienta de gestión ambiental, razón por la que el estudio de las mismas se ubica dentro del espacio de interés del manejo y conservación de los recursos naturales *(Ospina-Álvarez et Peña, 2004; Castiñeira, 2011).*

Se ha observado que en las algas se produce una bioacumulación de los elementos presentes en el medio donde crecen o se cultivan, llegando a constituir entre el 5% y el 20% de su materia seca *(Luján-de Fabricius, 2000; Castiñeira, 2011).* Por este motivo se han empleado como bioindicadores de contaminación por metales pesados *(Ospina-Álvarez et Peña, 2004)* y compuestos orgánicos *(Carafa et al., 2007; García-Rodríguez et al., 2012).*

Desde muy antiguo el hombre emplea las algas con usos muy diversos. Su gran diversidad y sus múltiples cualidades las hacen susceptibles de ser utilizadas en alimentación, agricultura, cosmética y medicina. Galicia gracias a su costa tan recortada y a su situación geográfica, es extraordinariamente rica y variada en especies de algas marinas. Esto, junto con las cada vez más importantes aplicaciones que tienen en nuestra vida actual, hace que cobren especial interés.

El uso de las algas en agricultura es tradicional en las zonas costeras. Los agricultores recolectan los arribazones, que son algas depositadas en las playas por el agua del mar, para incorporarlos como abono en los campos de cultivo de hortalizas. Un ejemplo es el uso de cianofíceas edáficas como fertilizantes de los campos de arroz, debido a su capacidad para fijar nitrógeno atmosférico.

Desde el punto de vista nutricional, cabe destacar su gran riqueza en vitaminas y minerales. Algunos de los minerales que contienen, como el calcio, magnesio, hierro, fósforo, potasio o yodo, se encuentran en la cantidad suficiente para cubrir un alto porcentaje de la cantidad que se recomienda ingerir diariamente. Son alimentos que aportan gran diversidad y cantidad de vitaminas, como vitaminas A y C y vitaminas del

grupo B. Algunas son muy ricas en proteínas, por lo que se recomienda su inclusión tanto en las dietas vegetarianas como en aquellas pobres en proteínas animales. Además son ricas en fibra y fitocoloides, sustancias que actúan favoreciendo la movilidad intestinal y de efecto saciante, lo que las hace adecuadas en dietas de adelgazamiento. También son recomendadas en dietas hipocalóricas debido a que son pobres en grasas e hidratos de carbono. Además, sus sabores, texturas y colores las hacen muy versátiles en la preparación de alimentos.

Por todo ello, presentan una gran aplicación en la industria alimentaria. Se pueden utilizar como tales en la alimentación humana (nori, wakame, etc.) o animal, aunque los productos obtenidos de las mismas, entre los que destacan el agar, el carragén y la algina, son los que se emplean principalmente en este tipo de industria. El agar y carragén son polisacáridos complejos que se obtienen de diversas algas rojas y son muy apreciados por su viscosidad y estabilidad. La algina o ácido algínico se obtiene de las algas pardas, sobre todo de especies de Laminaria, y es muy utilizado por su viscosidad y consistencia *(Braune et Guiri, 2011; Castiñeira, 2011).*

En la fabricación de cosméticos está muy extendida la utilización de determinados extractos de algas con objeto de mejorar las cualidades mecánicas del producto. Además, las algas contienen moléculas que participan activamente en los efectos beneficiosos que ofrecen determinados cosméticos *(Castiñeira, 2011).*

Otra aplicación importante de las algas es su empleo en la industria farmacéutica para la elaboración de medicamentos (contra la diabetes, arteriosclerosis, como antibióticos) y en odontología para la fabricación de moldes dentales, dentífricos, etc. Actualmente se estudian moléculas presentes en las algas que son activas en procesos tumorales, en procesos de regulación del colesterol y otras enfermedades o alteraciones comunes *(www.portomuiños.com).*

Esta relación entre la industria y las algas ha provocado una gran demanda de esta materia prima, por lo que las cofradías de pescadores comienzan a incorporar estos organismos en sus planes de explotación e incluso existen cultivos de algunas especies *(Braune et Guiri, 2011; Castiñeira, 2011).*

5.2. Mariscos: mejillón

Los mejillones son moluscos bivalvos cuyo cuerpo está constituido por dos partes: una externa, dura, la "concha" y otra interna, blanda, el "cuerpo" o "vianda". La concha de mejillón actúa como escudo y se encuentra formada por dos válvulas iguales. El cuerpo está constituido por dos lóbulos que a modo de abrigo abrazan y encierran las vísceras del animal. En sus bordes libres se encuentra una sinuosa banda de color violeta oscuro, la cual emite prolongaciones que se entrelazan constituyendo una

especie de filtro para impedir que puedan entrar partículas de tamaño excesivamente grande a su interior *(Figueras, 2007).* El mejillón se encuentra firmemente unido al sustrato mediante una maraña de hilos córneos denominada "el biso", cuyo origen es la secreción por la "glándula del biso" de una sustancia que se endurece al entrar en contacto con el agua.

Con respecto a su hábitat, el mejillón se encuentra en una amplia gama de hábitats, desde zonas intermareales hasta zonas totalmente sumergidas, con un variado abanico de temperaturas y salinidad, lo que explica su amplia distribución geográfica.

En cuanto a su alimentación, los mejillones son animales filtradores que se alimentan ingiriendo principalmente materia orgánica en forma de partículas inertes (detritos) y fitoplancton (microalgas o plancton vegetal). En menor medida consumen bacterias y también son capaces de absorber directamente sustancias orgánicas que se encuentran disueltas en el agua de mar. Debido a esto, se crían siempre en zonas ricas en plancton *(Durán-Neira et al., 1990).*

Se calcula que un mejillón es capaz de filtrar entre 0,5 y 7 litros de agua a la hora, por lo que desde que mide 20 milímetros hasta que alcanza 8 centímetros pueden pasar por su interior entre 20 y 23 metros cúbicos de agua. Traducido a una batea, constituyen más de 150.000 metros cúbicos diarios.

Los bivalvos marinos son unos extraordinarios indicadores de la calidad del agua debido a su sensibilidad a los cambios del medio en el que viven, ya que se caracterizan por presentar elevada sensibilidad a contaminantes orgánicos y alta tasa de acumulación de los mismos; además son estacionarios, presentan una amplia distribución geográfica y normalmente son la especie dominante en su hábitat *(Binelli et al., 2006; Damásio et al., 2010).* Todas estas cualidades los convierten en organismos idóneos para llevar a cabo programas de monitorización ambiental *(Sabater, 2005).*

La especie de mejillón presente en Galicia, tanto el mejillón salvaje capturado mediante marisqueo, como el mejillón cultivado mediante acuicultura, es la especie *Mytilus galloprovincialis.* Según la FAO (Organización de las Naciones Unidas para la Agricultura y la Alimentación), el cultivo de mejillón *Mytilus galloprovincialis* es el de mayor producción en la acuicultura de España y de los más importantes en Europa, donde en 2011 se superó una producción de más de 620.000 toneladas *(www.fao.org).*

Además, Galicia es el mayor productor de mejillón de Europa, y la industria de pescado y marisco es muy importante en esta área, puesto que exportan sus productos a todo el mundo. Por ello, el estudio de contaminantes en pescados y productos derivados de la pesca es de gran importancia para Galicia, tanto a nivel económico como medioambiental *(Durán-Neira et al., 1990).*

Desde el punto de vista nutricional, el mejillón es rico en proteína de calidad y de fácil digestión, siendo por ello muy beneficioso para la salud. Contiene muy poca grasa y elevada cantidad de ácidos grasos omega-3, que son cardioprotectores y regulan los niveles de triglicéridos en sangre, contribuyendo de esta manera a una reducción del riesgo de enfermedades cardiovasculares y a una disminución del nivel de colesterol. Además, es rico en vitaminas del complejo B y en minerales, destacando su elevado aporte en hierro y además de muy fácil asimilación, lo cual lo convierte en un elemento fundamental en dietas para personas con anemia ferropénica *(CESNID, 2008; Palma et al., 2008).*

Aunque las propiedades medicinales del mejillón están relacionadas con sus propiedades nutricionales, hace algunos años se han descubierto otras propiedades medicinales debidas a su elevado contenido en glucosaminas, que son el antiinflamatorio natural más potente, resultando de especial utilidad en procesos articulares como reuma, artritis y artrosis *(www.galiat6mas7.com).*

Otros de los usos del mejillón residen en su concha. Galicia produce anualmente unas 256.000 toneladas de este bivalvo, que generan 80.000 toneladas de residuos que en ocasiones se acumulan en zonas poco acondicionadas para este propósito, creando un efecto visual y olfativo indeseado, con graves perjuicios también desde el punto de vista ambiental. Por ello se han realizado una serie de estudios encaminados a la valorización de estos residuos con fines agrarios, forestales e incluso industriales (construcción de carreteras) *(Paz-Ferreiro et al., 2012).*

La concha de mejillón está constituida, fundamentalmente, por carbonato cálcico y por pequeñas cantidades de otros elementos como nitrógeno, azufre, fósforo, potasio y magnesio, por lo que se puede emplear para incrementar la fertilidad de suelos ácidos, como es el caso de Galicia. Así, investigadores de la Universidad Politécnica de Madrid demostraron que el uso de conchas de mejillón en suelos ácidos produce no sólo un incremento del pH, sino también una disminución de la cantidad de aluminio, por lo que puede emplearse con fines de biorremediación *(Paz-Ferreiro et al., 2012).*

5.3. Pescados: trucha

Actualmente existe un reconocimiento generalizado de que la producción de pescados y mariscos mediante pesca de captura ha alcanzado su pico máximo, mientras que la producción de alimentos marinos mediante acuicultura será cada vez más importante *(Lucas et al., 2012; Purser et Forteath, 2012).* Por otro lado, cada vez es mayor el interés público por la acuicultura; de hecho la producción acuícola mundial está aumentando mucho más rápidamente que la pesca de captura y la ganadería, que son las otras dos fuentes de proteína animal para la población mundial. Por ello, con el

fin de garantizar la seguridad alimentaria, el control de compuestos químicos que pueden originar problemas importantes en los peces criados en estas explotaciones adquiere cada vez mayor interés.

Los peces pueden incorporar plaguicidas a través de dos vías, absorción branquial desde el agua e incorporación a través de la cadena alimenticia. También se ha admitido la absorción a través de la piel, aunque esta forma de absorción es de mucha menor importancia *(García-Cambero et Soler-Rodríguez, 2005).* En el caso de los peces de acuicultura los herbicidas pueden acumularse a través de la alimentación que éstos reciben (piensos fabricados a partir de vestigios de otros peces de escaso valor comercial, o piensos vegetales que pueden contener restos de plaguicidas). Por otro lado, como ya se dijo anteriormente, algunos pesticidas se aplican directamente en los estanques donde se cultivan los peces para controlar el nacimiento de algas y eliminar organismos invertebrados. Además, algunos criaderos están situados en zonas cercanas a explotaciones agrarias, por lo que corren el riesgo de que los pesticidas utilizados para el cultivo de las tierras sean transportados a través del medio hasta llegar al agua donde se produce el cultivo acuícola *(Fernández-Álvarez et al., 2009; www.sciencedaily.com).* Todo ello ha traído como consecuencia que se hayan encontrado concentraciones más altas de contaminantes en pescados procedentes de acuicultura que en pescados procedentes de captura *(Cole et al., 2009).*

Por todo lo expuesto, en este trabajo se ha considerado de gran interés el estudio de pescados procedentes de acuicultura, siendo la trucha el pez seleccionado por tres razones fundamentales: en los últimos años se ha producido un fuerte incremento en la producción de salmónidos; la trucha es uno de los cuatro pescados seleccionados en la guía SANCO para estudiar la aplicabilidad de los métodos analíticos destinados a la determinación de pesticidas; y en tercer lugar, la FDA considera a la trucha de acuicultura como un pescado potencial para la acumulación de contaminantes químicos.

La principal especie de trucha cultivada en el mundo es la trucha arco iris (*Oncorhynchus mykiss*), un salmónido originario de la costa Pacífica de Norteamérica, que ha sido introducido en los ríos de todos los continentes. Al igual que el resto de salmónidos, es una especie anádroma migratoria, caracterizada por un elevado polimorfismo que ha llevado a la clasificación de un gran número de subtipos. La otra especie que también es objeto de cría es la trucha común (*Salmo trutta*) conocida también en España como reo o trucha de mar *(Blaginièri, 1999; www.cetusreports.com).*

En cuanto a su morfología, la trucha arco iris presenta un cuerpo alargado y fusiforme. Como todos los salmónidos, tiene una aleta dorsal adiposa, generalmente con

un borde negro. Una banda irisada rosácea longitudinal le marca los flancos; por encima es de color azul-verdoso y por debajo, el vientre es color gris plateado o blanquecino.

La trucha arco iris presenta unas características de cultivo ventajosas respecto del resto de especies.

- Tolera un amplio rango de temperaturas lo que permite su adaptación a una gran variedad de cursos de agua en regiones de diferentes climatologías.
- Tiene una buena tasa de crecimiento. Al ser una especie de aguas frías, no ve significativamente alterado su crecimiento durante el invierno.
- Su reproducción en cautividad es sencilla. Sus larvas son suficientemente grandes como para que la primera alimentación pueda basarse en piensos preparados.
- Resiste bien las condiciones de cultivo intensivo.

Con respecto a sus propiedades nutricionales, la trucha se considera un pescado semigraso. Aporta proteínas de alto valor biológico, de manera que contiene todos los aminoácidos esenciales. También aporta vitaminas A, B_1, B_2 y B_3 y minerales como hierro, magnesio, potasio, fósforo y zinc. Es un pescado especialmente rico en ácidos grasos omega-3, los cuales ayudan a prevenir enfermedades cardiovasculares. Gracias a su bajísimo aporte calórico ayuda a disminuir el sobrepeso y la obesidad, empleándose en dietas de adelgazamiento. Su contenido en sal es bajo, de forma que su consumo está recomendado en personas con hipertensión arterial *(Maraver, 2013).*

La alimentación de la trucha de acuicultura está basada en piensos para peces carnívoros, con elevadas concentraciones energéticas y altos porcentajes de proteína (35-40%), siendo las principales materias primas utilizadas en la elaboración de estos piensos la harina de pescado, cereales, y aceites de pescado *(Maraver, 2013).*

6. LEGISLACIÓN

Las triazinas son uno de los grupos de herbicidas más utilizados en todo el mundo. Actualmente están considerados, aunque en menor grado que otros pesticidas, medioambientalmente peligrosos debido a su persistencia, toxicidad y capacidad de bioacumulación. Así la Atrazina, ampliamente utilizada, fue vetada en Italia y Alemania en el 2000 y en Francia dos años más tarde; sin embargo, la Propazina ya había sido retirada del mercado en Estados Unidos en 1994 *(Thurman et al., 1994; Sabik et al., 2000).*

La prevención de la contaminación ha ido en aumento en los últimos años, por lo que diversos organismos nacionales e internacionales han establecido límites máximos de residuos (LMRs) de estas sustancias en aguas y alimentos con el fin de proteger la

salud humana y el medioambiente. Así, la agencia de protección ambiental norteamericana (EPA) considera la Atrazina y la Simazina dentro del grupo de disruptores endocrinos *(Environmental Protection Agency of United States, 2009).* Por otra parte, el ecosistema acuático implica la estimación de la calidad del agua y de los organismos biológicos. Además, cabe destacar que la conservación y el uso sostenible del medio marino constituyen factores importantes para la prosperidad económica, el bienestar social y la calidad de vida.

Las primeras directivas en las que aparecen legislados los herbicidas en aguas son la *Directiva 80/778/CE del 15 de Julio de 1980* y la *Directiva 98/83/CE del 3 de Noviembre de 1998, relativas a la calidad del agua de consumo humano* en las que se establece un límite para cada plaguicida de 0,10 µg/L y para el total de plaguicidas 0,50 µg/L, donde los plaguicidas incluyen, entre otros, los herbicidas orgánicos. Estos mismos valores son los establecidos en la legislación española en el *Real Decreto 1138/1990 del 14 de septiembre*, por el que se aprueba la reglamentación Técnico-Sanitaria para el abastecimiento y control de calidad de las aguas potables de consumo público.

En el año 2000, la *Directiva 2000/60/CE del Parlamento Europeo y del Consejo* establece un marco comunitario de actuación en el ámbito de la política de aguas, e incluye Atrazina y Simazina como sustancias peligrosas. Posteriormente la *Decisión nº 2455/2001/CE del Parlamento Europeo y del Consejo del 20 de noviembre de 2001,* aprueba una lista de 33 sustancias prioritarias en el ámbito de la política de aguas, entre las que se incluyen Atrazina y Simazina.

Más adelante, en la *Directiva 2008/105/CE del Parlamento Europeo y del Consejo del 16 de diciembre de 2008,* relativa a las normas de calidad ambiental en el ámbito de la política de aguas, se establece una concentración máxima admisible (CMA) en aguas superficiales, de 2 y 4 µg/L para Atrazina y Simazina, respectivamente. Por otro lado, esta Directiva también insta a los Estados Miembros a establecer parámetros de calidad a nivel nacional para estos contaminantes en sedimentos y biota. Recientemente, la *Directiva 2013/39/UE del Parlamento Europeo y del Consejo del 12 de agosto de 2013,* que es la normativa que se encuentra actualmente en vigor, incorpora la Terbutrina a la lista de sustancias prioritarias, estableciendo una concentración máxima de 0,34 µg/L en aguas superficiales.

Siguiendo la misma línea, la *Directiva Marco de Estrategia Marina 2008/56/CE* establece entre los descriptores de buena salud ambiental que "las concentraciones de contaminantes se encuentren en niveles que no den lugar a efectos de contaminación y que los contaminantes presentes en pescados y otros productos pesqueros dirigidos al consumo humano, no superen los niveles establecidos por la normativa comunitaria."

Con respecto a matrices y alimentos de origen vegetal, la primera directiva es la *Directiva 86/362/CE del 24 de julio de 1986*, relativa a la fijación de contenidos máximos para los residuos de plaguicidas sobre y en los cereales. En ella se establece un límite de 0,1 mg/kg de Atrazina en cereales. Posteriormente, la *Directiva 90/642/CE del consejo de 27 de noviembre de 1990*, relativa a la fijación de los contenidos máximos de residuos de plaguicidas en determinados productos de origen vegetal, incluidas las frutas y hortalizas, establece para Atrazina un límite de 0,1 mg/kg.

Aun así, seguían existiendo diferencias en los límites máximos nacionales de residuos de plaguicidas que podían crear obstáculos al comercio de los productos entre los Estados miembros y entre terceros países y la Comunidad. Por tanto, en aras de la libre circulación de mercancías, de la equidad de las condiciones de competencia entre los Estados miembros, así como de un nivel elevado de protección de los consumidores, surge *el Reglamento 396/2005/CE del Parlamento Europeo y del Consejo de 23 de febrero de 2005*, relativo a los límites máximos de residuos de plaguicidas en alimentos y piensos de origen vegetal y animal.

En el caso de las algas, los primeros límites se establecen en *el Reglamento 149/2008/CE del Parlamento Europeo y del Consejo de 29 de enero de 2008*, que modifica el *Reglamento 396/2005/CE*. En este Reglamento se establecen para las algas límites de 0,10 y 0,05 mg/kg para Simazina y Terbutilazina respectivamente. Posteriormente el límite fijado para la Simazina se redujo a 0,01 mg/kg a través del *Reglamento 310/2011/CE del Parlamento Europeo*. En el caso de la Atrazina, no existe legislación para algas, siendo su límite máximo admisible en la mayoría de vegetales terrestres de 0,05 mg/kg.

En cuanto a alimentos de origen animal, aunque la legislación Europea establece límites para Simazina y Terbutilazina en organismos terrestres, todavía no tiene establecidos límites máximos de residuos de plaguicidas en organismos acuáticos. Sin embargo la FDA establece un nivel de tolerancia de 12 mg/kg para la Simazina en pescados y productos de la pesca *(Food and Drug Administration, 2011).*

Debido a que los límites fijados son muy restrictivos, muchas metodologías que se están utilizando en la actualidad no permiten alcanzar los límites establecidos, poniendo de manifiesto la necesidad de disponer de métodos de análisis validados, sensibles y selectivos que permitan su aplicación en programas de monitorización y control. Respecto a esto, la *Directiva 2009/90/CE de la Comisión de 31 de julio de 2009*, establece los criterios mínimos que se deberán aplicar a los métodos de análisis para el seguimiento del estado de las aguas, sedimentos y seres vivos, así como las normas dirigidas a demostrar la calidad de los resultados analíticos.

Por otro lado, en 1997, la Comisión Europea publicó un documento guía (SANCO Guidelines), para el control de la calidad analítica y los procedimientos de validación de los métodos para el análisis de residuos de pesticidas en alimentos y piensos, siendo la edición publicada en Noviembre de 2015 (*SANTE/11945/2015)* la que se encuentra actualmente en vigor.

7. MÉTODOS DE EXTRACCIÓN

Los herbicidas triazínicos, al igual que otros contaminantes persistentes, se acumulan en una gran variedad de matrices en las que los niveles de concentración permitidos por las diferentes legislaciones son cada vez menores, llegando a niveles traza de concentración. Además, en los últimos años se han detectado un gran número de pesticidas y de sus metabolitos en muestras de alimentos y medioambientales. Todo ello ha traído como consecuencia que durante la última década se haya logrado un enorme progreso en los métodos de preparación de muestra que pueden ser aplicados al análisis de contaminantes. Sin embargo, todavía es necesario avanzar en los métodos de extracción de plaguicidas, ya que hoy en día el desafío en el análisis de pesticidas es el desarrollo de métodos de preparación de muestra eficaces, económicos y selectivos, con el fin de satisfacer los requisitos de sensibilidad.

La preparación de la muestra para el análisis cromatográfico es una de las etapas más críticas en el proceso analítico. El principal problema es la separación de los analitos de interés de otros componentes de la matriz, debido a una extracción ineficaz, pérdida de muestra y bajos niveles del analito en la muestra.

7.1. Matrices acuosas

El método clásico de extracción de triazinas y sus productos de degradación en aguas superficiales es la extracción líquido-líquido (LLE) *(Retamal et al., 2013).* Los principales inconvenientes de este método de extracción son el elevado consumo de disolvente, un tiempo largo de extracción y la necesidad de una etapa de limpieza posterior para eliminar posibles sustancias interferentes. Como alternativa a la extracción líquido-líquido, la técnica más empleada para la determinación de estos analitos en muestras acuosas es la extracción en fase sólida (SPE) usando diferentes adsorbentes *(Kueseng et al., 2009; van Pinxteren et al., 2009; Bagheri et al., 2010; Benvenuto et al., 2010; Dujaković et al., 2010; García-Galán et al., 2010; Katsumata et al., 2010; Postigo et al., 2010; Lissalde et al., 2011; Akdogan et al., 2013*).

En los últimos años, con el fin de minimizar el consumo de disolventes orgánicos de acuerdo a los principios de la Química Verde, se han desarrollado técnicas de micro-extracción para la determinación de herbicidas triazínicos en muestras de

agua, como son la microextracción en fase sólida (SPME) *(Mohammadi et al., 2009; Djozan et al., 2010; Passeport et al., 2010),* la micro-extracción en fase líquida (LPME) *(Hu et al., 2009a; Trić-Petrović et al., 2010),* la extracción con barras de agitación magnética (SBSE) *(Portugal et al., 2008),* la micro-extracción líquido-líquido dispersiva (DLLME) *(Nagaraju et al., 2007; Zhou et al., 2009; Wang et al., 2011a; Sanagi et al., 2012),* la microextracción líquido-líquido-sólido (LLSME) *(Hu et al., 2009a)*, la microextracción en gota o "Single Drop" (SDME) *(Ye et al., 2007)* y la microextracción en fase líquida en fibra hueca (HF-LPME) *(Trić-Petrović et al., 2010).* Además, algunas de estas técnicas han sido empleadas para la extracción de las triazinas y alguno de sus productos de degradación, como SPME *(Moliner-Martínez et al., 2015),* SBSE *(Sánchez-Ortega et al., 2009)* y la microextracción en fase sólida dispersiva (DMSPE) *(Chen et al., 2015).*

Estas técnicas tienen ciertas ventajas sobre SPE, pero también presentan algunos inconvenientes ya que algunas de ellas muestran baja sensibilidad para los analitos estudiados *(Mohammadi et al., 2009)* o baja eficiencia de extracción *(Zhou et al., 2009; Wang et al., 2010)* y otras son muy laboriosas *(Hu et al., 2009a; Trić-Petrović et al., 2010).*

En este trabajo se han empleado dos de estas técnicas, la extracción en fase sólida (SPE) y la microextracción líquido- líquido dispersiva (DLLME).

7.1.1. Extracción en fase sólida

Esta técnica consiste en preconcentrar y aislar los analitos sobre un soporte sólido eluyendo posteriormente los compuestos de interés. Se basa en la diferente afinidad que presenta el analito por una fase sólida o por la muestra líquida. La muestra se pone en contacto con la fase sólida quedando retenidos los compuestos que tengan propiedades químicas similares a las del adsorbente. En algunos casos, se realiza el lavado del cartucho para eliminar posibles sustancias interferentes y, posteriormente, se lleva a cabo la elución de los analitos con un pequeño volumen de un disolvente que tenga afinidad por los analitos retenidos *(Abbas et al., 2015).* Este método es el más empleado para determinar triazinas y sus productos de degradación en muestras acuosas y permite realizar simultáneamente extracción, purificación y concentración de la muestra.

La forma habitual de trabajo es aquella en la que se coloca la fase sólida (adsorbente) en un cartucho de vidrio o de polietileno, similar al cuerpo de una jeringa. Se distinguen tres etapas: en primer lugar, el adsorbente es acondicionado con un disolvente de propiedades similares a la muestra; a continuación un determinado volumen de muestra se pasa a través de él quedando así los analitos retenidos.

Finalmente, después de una etapa de lavado para eliminar posibles compuestos interferentes, los analitos son eluidos con un disolvente adecuado. La elección del adsorbente a utilizar dependerá del analito, de su nivel de concentración y del disolvente en el que se encuentre.

Otra alternativa a los cartuchos, son los discos de extracción, en los que el adsorbente se encuentra en una red de microfibras de politetrafluoroetileno (PTFE) formando un disco de 0,5 mm de espesor y de distintos diámetros. Su principal ventaja es la posibilidad de preconcentrar los analitos a caudales mayores que los empleados en los cartuchos ya que no hay riesgos de formación de caminos preferentes y la transferencia de masa es más eficaz debido al menor tamaño de partícula utilizado en su fabricación.

Entre los adsorbentes empleados para la extracción de triazinas en aguas mediante SPE, el adsorbente apolar más utilizado es el octadecilsilano (C_{18}) debido a su capacidad para retener tanto analitos apolares como moderadamente polares. Además, ha sido uno de los primero adsorbentes empleado para la extracción de triazinas *(Carabias-Martínez et al., 2003; Lekkas et al., 2004; Berzas-Nevado et al., 2007; Zhao et al., 2008; Brix et al., 2009; van Pinxteren et al., 2009; Beale et al., 2010; Bester et Lamani, 2010; Kueseng et al., 2009; Dujaković et al., 2010; Portolés et al., 2011; Akdogan et al., 2013; Terzoupoulou et al., 2015; Rocha et al., 2015).*

En el caso de los productos de degradación, que contienen grupos funcionales polares, este tipo de adsorbente no es muy adecuado necesitándose adsorbentes polares para su extracción *(Papadopoulos et al., 2007).* Así, estudios realizados empleando C_{18} han mostrado que, mientras la recuperación obtenida para las triazinas era aceptable, sus productos de degradación presentaron recuperaciones muy bajas *(Papadopoulos et al, 2007; Berzas-Nevado et al., 2007; Portolés et al., 2011; Barchanska et al., 2012; Terzopoulou et al., 2015).*

Por otra parte, también se han empleado adsorbentes basados en carbón. La principal propiedad del carbón es su elevada superficie específica que conlleva una alta capacidad de adsorción y fuerte interacción con los anillos aromáticos de las moléculas orgánicas, por ello tiene aplicación en la determinación de triazinas. Para la extracción de triazinas en agua se han empleado adsorbentes basados en carbón tales como Carbón Negro Grafitizado (GCB) conocido comercialmente como carbopack B *(Curini et al., 2000)* y carbón de bambú *(Zhao et al., 2008)*. En el caso de los productos de degradación también se han utilizado cartuchos de GCB *(Sabik et al., 2000; Papadopoulos et al., 2007).*

Para la obtención de adsorbentes selectivos se ha empleado la técnica de impresión molecular "*molecular imprinting*", la cual permite la preparación de

polímeros de huella molecular (MIPs), con puntos de unión selectivos capaces de reconocer a una determinada molécula. Para la extracción de triazinas en aguas, se han empleado adsorbentes MIPs de Terbutilazina *(Ferrer et al., 2000)*, Propazina *(Guzzella et al., 2006)*, Atrazina *(Sambe et al., 2007; Djozan et Ebrahimi, 2008; Kueseng et al., 2009)* o MIPs de esferas magnéticas *(Hu et al., 2009b)*. Sin embargo, este tipo de adsorbentes no se han empleado para la extracción de los productos de degradación.

Actualmente existe una gran variedad de adsorbentes para la extracción en fase sólida de triazinas y/o sus principales productos de degradación. Los más utilizados son los adsorbentes comerciales hidrofílicos obtenidos por copolimerización como el Oasis HLB, que es un polímero macroporoso poli (N-vinilpirrolidina-divinilbenceno) (PVP-DVB) que ha sido empleado por Navarro et al., *(2010)* y Dujaković et al., *(2010)* para el análisis de triazinas y por otros autores para analizar triazinas y alguno de sus metabolitos en aguas *(Benvenuto et al., 2010; Huff et Foster, 2011; Lissalde et al., 2011; Rocha et al., 2011; Barchanska et al., 2012; Hurtado-Sánchez et al., 2013; Masiá et al., 2013; Kalogridi et al., 2014a; Terzoupoulou et al., 2015).* Entre las ventajas de este adsorbente se encuentra la posibilidad de extraer compuestos polares y apolares, limpieza de matrices complejas y elevada capacidad y eficacia a la hora de eliminar posibles interferencias *(Gilart et al., 2014)*. Otros adsorbentes poliméricos hidrofílicos utilizados para la extracción en fase sólida de triazinas son Bond Elut Plexa o Strata-X *(Gilart et al., 2014)*, nanofibras de Nylon 6/polipirrol *(Yang et al., 2015)* y el grafeno *(Wu et al., 2015).*

En los estudios de triazinas se han empleado otros adsorbentes tales como poli (estireno-divinilbenceno), comercialmente conocido como LiChrolut EN *(Curini et al., 2000).* Este adsorbente también se ha utilizado en estudios en los que se incluye alguno de los productos de degradación *(Carabias-Martínez et al., 2006; Barchanska et al., 2012).* Por otra parte, también se han utilizado cartuchos rellenos de partículas esféricas macroporosas rígidas de poliestireno y divinilbenceno (PLRP-s) *(Köck et al., 2010; Postigo et al., 2010; Köck-Schulmeyer et al., 2012; Hurtado-Sánchez et al., 2013)* o estireno divinil benceno, conocido comercialmente como Bond Elut-ENV *(Berzas-Nevado et al., 2007).*

Otro tipo de material empleado para la extracción de triazinas y alguno de los productos de degradación es un adsorbente de intercambio iónico, Oasis MCX. Es un adsorbente en modo mixto, poli (N-vinilpirrolidina-divinilbenceno) de intercambio catiónico-fase reversa (MCX). Este adsorbente presenta muy buenos resultados para los productos de degradación que han estudiado diferentes autores *(Papadopoulos et al., 2007; Benvenuto et al., 2010; Li et al., 2013).* Además, se han empleado copolímeros de poliestireno divinilbenceno funcionalizado con un intercambiador de cationes (SCX)

(Sabik et al., 2000), Amberlita XAD-4 *(Adkogan et al., 2013)* y Oasis MAX *(Zhang et al., 2014).*

Sin embargo, el adsorbente que ha mostrado una mejor capacidad para retener tanto las triazinas como algunos de los productos de degradación es el Oasis HLB *(Gervais et al., 2008; Benvenuto et al., 2010).*

Respecto a los eluyentes, los más utilizados para realizar la elución de las triazinas en aguas son metanol *(Beale et al., 2009; Kueseng et al., 2009; Bester et Lamani, 2010; See et al., 2010; Akdogan et al., 2013)* y acetonitrilo *(Zhou et al., 2006; Zhao et al., 2008; Bester et Lamani, 2010).* Otros eluyentes empleados consisten en mezclas de metanol con otro disolvente tales como metanol:acetona (1:1) *(Muller et al., 2008),* metanol:acetonitrilo (1:1) *(Ruggieri et al., 2005),* metanol al 10% (v/v) en metil-terbutil-éter (MTBE) *(Huff et Foster, 2011),* metanol:diclorometano (1:1) *(Masiá et al., 2013),* metanol:acetato de etilo (1:1) *(Carabias-Martínez et al., 2002)* o (75:25) *(Lissalde et al., 2011)* y mezcla de metanol, acetona, acetato de etilo (2:2:1) *(Carafa et al., 2007).* También se han empleado mezclas de acetonitrilo con otros disolventes tales como acetonitrilo:agua (1:1) *(van Pinxteren et al., 2009)* y acetonitrilo:diclorometano (1:1) *(Gervais et al., 2008)* y otros disolventes como acetato de etilo solo *(Carabias-Martínez et al., 2003),* en proporción (1:1) con diclorometano *(Navarro et al., 2010; Portolés et al., 2011),* o en proporción (1:9) con metil-terbutil-éter *(Beale et al., 2010).*

Los métodos de SPE off-line, generalmente requieren un elevado volumen de muestra y un tiempo de análisis largo *(Loos et al., 2010).* Sin embargo, cuando se emplea SPE on-line, la cantidad de disolvente necesaria para la extracción es mínima, la preparación de la muestra es rápida y los volúmenes de muestra empleados son mucho menores *(Trenholm et al., 2009).* SPE on-line también presenta algunas desventajas como la complejidad del montaje del sistema de válvulas, la falta de flexibilidad en comparación con SPE off-line y las posibles interferencias que puede suponer cargar toda la muestra de la que se van a extraer los analitos de interés.

Sin embargo, la mayoría de estos problemas se han resuelto debido a los avances en los sistemas automatizados para SPE on-line, al empleo de programas de software integrado y flexible, así como al uso de detectores de espectrometría de masas para una mejor selectividad. Por ello, en los últimos años se ha incrementado el uso de SPE on-line acoplada a cromatografía líquida con espectrometría de masas, encontrándose algunos métodos para el análisis de triazinas y alguno de sus productos de degradación en agua *(Postigo et al., 2010; Köck-Schulmeyer et al., 2012; Hurtado-Sánchez et al., 2013).*

7.1.2. Microextracción líquido-líquido dispersiva

Las técnicas de microextracción líquido-líquido (LLME), son técnicas recientes que se basan en la miniaturización de la extracción líquido-líquido, utilizando volúmenes muy pequeños del disolvente de extracción (< 1 mL) *(López-Darias et al., 2010; Wang et al., 2011a)*. Las más utilizadas son la microextracción en gota de disolvente (SDME), la microextracción líquido-líquido dispersiva (DLLME) y la microextracción líquido-líquido en fibra hueca (HF-LLME).

La primera técnica de microextracción líquido-líquido surgió en 1995, en la que se utilizó un sistema de microextracción empleando una gota de disolvente *(Liu et al., 1995)*. Posteriormente, teniendo en cuenta que el área de contacto entre la fase orgánica y la fase acuosa podría aumentar si en lugar de una gota se forma una nube de gotas de menor tamaño, mejorando de este modo el proceso de extracción, se ha empleado la técnica DLLME *(Rezaee et al., 2006)*.

La técnica DLLME consiste en la extracción de analitos de muestras acuosas utilizando una mezcla apropiada entre un disolvente de extracción de elevada densidad e inmiscible con el agua, y un disolvente dispersante polar y miscible con el agua. La mezcla de disolventes se pone en contacto con la muestra obteniéndose una disolución turbia que asegure una superficie de contacto elevada. El extractante representa sólo entre el 1-3% del total del volumen de la mezcla de extracción formando una nube de diminutas gotas que, debido al elevado contacto superficial, permite alcanzar el equilibrio rápidamente. Por último se centrifuga y se juntan las pequeñas gotas en una sola de mayor tamaño. Esta última etapa del proceso es el momento en el cual se produce la separación de las fases, quedando los analitos retenidos en la fase por la que presentan una mayor afinidad. La gota generada se puede recoger con una microjeringa. En la **figura 1.9.** se muestra el proceso.

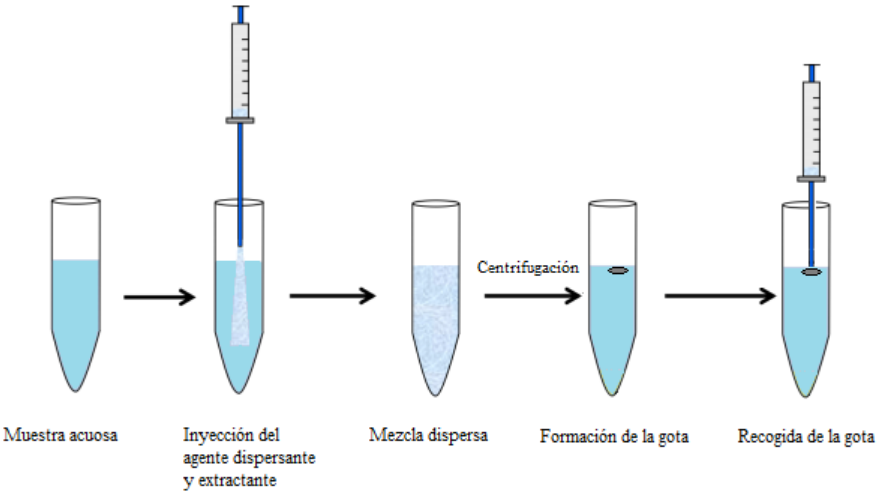

Figura 1.9. Etapas de la microextracción líquido-líquido dispersiva (DLLME)

Los agentes extractantes más utilizados son disolventes clorados como diclorometano, clorobenceno o tetracloroetileno, o alcoholes como octanol, decanol o dodecanol *(Wang et al., 2011a; Sanagi et al., 2012).* Entre los agentes dispersantes, los más empleados son acetonitrilo, acetona o metanol *(López-Darias et al., 2010; Zhang et al., 2011).*

Las principales ventajas de esta técnica de extracción son la simplicidad de operación, su bajo coste, el pequeño volumen de muestra empleado y el elevado factor de enriquecimiento conseguido. Además, la extracción puede completarse en varios segundos, consiguiendo una etapa de extracción muy rápida *(Ojeda et Rojas, 2009; Wang et al., 2010).*

El principal problema de esta técnica de extracción es la selección adecuada de la mezcla de disolventes, así como la proporción entre ambos, puesto que pueden producirse pérdidas de los analitos si ésta no es la adecuada. Puede evitarse sustituyendo la adición del dispersante por una etapa de agitación para obtener la formación de la disolución turbia *(Zhang et al., 2011).* En esta memoria se realiza la extracción con la técnica DLLME con agitación vórtex.

En los últimos años, esta técnica ha sido muy empleada para el análisis de diferentes contaminantes orgánicos en matrices ambientales como hidrocarburos aromáticos policíclicos *(Rezaee et al., 2006)* o disruptores endocrinos *(López-Darias et al., 2010).* Entre los contaminantes que se han extraído mediante esta técnica, también se encuentran las triazinas *(Nagaraju et Huang, 2007; Zhou et al., 2009; Wang et al., 2011a; Sanagi et al., 2012).*

Nagaraju et Huang *(2007),* han determinado ocho triazinas entre las que se encuentran Ametrina, Atrazina, Prometrina, Propazina y Simazina en muestras acuosas utilizando esta técnica extractiva y su posterior análisis con GC-MS. Para realizar la extracción, escogieron clorobenceno como disolvente extractante, y acetona como agente dispersante. Esta técnica también se ha utilizado para determinar Atrazina y Simazina en agua de grifo, subterránea y de embalses, utilizando metanol como agente dispersante y tetracloruro de carbono como disolvente de extracción *(Zhou et al., 2009).* Wang et al. *(2011),* la han empleado para la extracción de Ametrina, Atrazina, Prometrina y Simazina de muestras de agua y suelo, empleando cloroformo y acetonitrilo como disolvente de extracción y agente dispersante respectivamente. Por otra parte, se han comparado diferentes disolventes de extracción (1-dodecanol, 1-undecanol y n-hexadecano) y distintos dispersantes (acetona, acetonitrilo y metanol) para la determinación de Atrazina, Cianazina y Simazina en muestras de agua y caña de azúcar. Los mejores resultados fueron obtenidos empleando 1-undecanol como extractante y acetonitrilo como dispersante *(Sanagi et al., 2012).*

Otra de las técnicas de microextracción líquido-líquido empleada para el análisis de triazinas es la SDME. Existen diferentes modalidades que se clasifican atendiendo al número de fases que intervienen en el equilibrio. La modalidad más empleada es la microextracción en gota por inmersión directa (DI-SDME), donde una gota de disolvente situada en la punta de la aguja de una microjeringa se pone en contacto directo con una muestra acuosa *(López-Darias et al., 2010).* Ye et al. *(2007),* utilizaron esta técnica para determinar triazinas en diferentes muestras de aguas obteniendo resultados satisfactorios.

La técnica HF-LLME fue presentada en 1999 por Pedersen-Bjergaard et Rasmussen como una alternativa más robusta a la microextracción en gota. Esta modalidad de microextracción consiste en impregnar los poros de una fibra hueca con un disolvente orgánico y puede realizarse en la modalidad de dos o tres fases. Cuando se trabaja en dos fases, los analitos se extraen desde la muestra acuosa a un disolvente orgánico inmiscible con el agua que está inmovilizado en los poros de la fibra hueca y en el interior de la fibra (disolución aceptora). La extracción se basa en la difusión pasiva por las constantes de distribución de los analitos. En la modalidad de tres fases, los analitos extraídos desde la muestra acuosa pasan a través de una fina capa de disolvente orgánico inmiscible con el agua que se encuentra inmovilizado en los poros de la fibra hueca y en el interior de la fibra se introduce una segunda fase acuosa aceptora. Como en el caso anterior, los analitos se extraen por difusión pasiva basada en sus coeficientes de distribución *(Pedersen-Bjergaard et Rasmussen, 1999).* Esta técnica se ha empleado para analizar triazinas en muestras complejas de agua, utilizando una membrana porosa formada por un polímero de impresión molecular y realizando el análisis con HPLC-DAD *(Hu et al., 2009a).*

Recientemente, Chen et al. *(2015)* utilizaron la técnica DMSPE en la determinación de un total de 30 triazinas (entre las cuales están Ametrina, Atrazina, Cianazina, Prometrina, Propazina, Simazina y Simetrina y los productos de degradación DEA, DIA y HA) en aguas de bebida y agua de grifo. El procedimiento consiste en mezclar la muestra con el adsorbente (PCX) en medio 0,1% de ácido fórmico y agitación vórtex. A continuación se trasvasa a una jeringa, la disolución obtenida en la extracción se desecha y los analitos son eluidos del adsorbente.

7.2. Matrices sólidas

Tal y como se comentó en el apartado anterior, la preparación de la muestra, fundamentalmente la etapa de extracción, es una etapa crítica sobre todo en lo que respecta a matrices complejas. En el caso de los sedimentos, éstos presentan una gran complejidad ya que están formados por organismos vivos, materia orgánica y partículas minerales *(García-Flores et Maza-Álvarez, 2002).* Respecto a las algas, en su

composición se encuentran diferentes compuestos interferentes que afectan al análisis de pesticidas, principalmente pigmentos como clorofilas y carotenoides, polisacáridos, ácidos grasos poliinsaturados, vitaminas y minerales, que frecuentemente son coextraídos *(Dhargalkar et Verlerkar, 2009; García-Rodríguez et al., 2010; Lorenzo et al., 2012)*. En relación a los tejidos de origen animal, el principal reto es minimizar el contenido lipídico de los extractos finales *(Sapozhnikova, 2015);* por ello la determinación de pesticidas se complica en estas matrices, debido al diferente contenido graso de los diferentes pescados y mariscos *(Negreira et al., 2013).*

Las metodologías más frecuentemente empleadas para la determinación de triazinas en matrices sólidas tanto de origen vegetal (algas) como animal (pescados y mariscos) y en sedimentos utilizan extracción con disolventes, desde técnicas convencionales tales como Soxhlet, agitación mecánica y ultrasonidos, hasta técnicas más recientes como extracción con energía de microondas (MAE) y extracción con disolventes a presión (PLE).

Sin embargo, estas técnicas necesitan añadir una etapa de purificación para disminuir la presencia de sustancias interferentes en el extracto final, con objeto de disminuir los límites de detección y evitar solapamientos en el análisis cromatográfico. Por ello estos métodos son costosos en términos de tiempo, de consumo de materiales, y de muestra, y el rendimiento es demasiado bajo para afrontar los retos del análisis moderno ambiental o de alimentos. En la **tabla 1.1.** se presenta una relación de trabajos recogidos en la literatura en los que se detallan las técnicas de extracción y purificación empleadas, indicándose también la técnica analítica utilizada.

Tabla 1.1. Resumen de las distintas técnicas de extracción utilizadas por otros autores para la determinación de triazinas

Matriz	Triazinas	Método de extracción	Purificación	Técnica de determinación	Referencia
Soxhlet					
Sedimento	Atrazina Simazina	5 g de muestra, extracción en continuo durante 24 horas con una disolución de metanol:diclorometano (2:1, v/v).	SPE en columna de florisil desactivada con 2% de agua.	GC-MS	*Galanopoulos et al., 2005*
Sedimento	Atrazina, Propazina, Simazina, Terbutilazina	1 g de sedimento, extracción durante 24 h con 100 mL de disolución hexano:acetona (1:1, v/v).	SPE, con 2 g de alúmina y elución secuencial con 20 mL de mezcla acetona:hexano (2:1, v/v) seguida de 20 mL de la misma mezcla (1:10, v/v).	GC-MS	*Villaverde et al., 2008*
Cereales	Atrazina, Prometrina, Simazina	2 g de muestra, extracción durante 2 h con 40 mL de metanol a 100ºC. Se repite el proceso 3 veces y se juntan las tres porciones.	---	HPLC-MS	*You et al., 2007*
Leche en polvo	Ametrina, Atrazina, Prometrina, Propazina, Simetrina	1 g de muestra, extracción durante 1 h utilizando como agente extractante 0,1% de agua en metanol.	SPE con cartuchos de Oasis MCX como adsorbente y elución con 10 mL de disolución de hidróxido amónico al 4% en metanol.	LC-MS/MS	*Fang et al., 2012*
Carne, frutas, vegetales	Atrazina, Simazina	5 g de muestra, extracción entre 6 y 8 horas utilizando acetona:hexano (1:1, v/v) .	SPE empleando 10 g de alúmina como adsorbente y 30 mL de cloruro de metileno:hexano (70:30, v/v) como eluyente.	GC-MS	*Rosenblum et al., 2001*
Agitación mecánica					
Sedimento	Ametrina, Atrazina, Prometrina	100 g de muestra, 100 mL de MeOH:ACN (1:1, v/v). Agitación vórtex durante 30 min.	---	HPLC-DAD	*Li et al., 2010*
Sedimento	DEHA, DET, DIA, DIHA, Terbutilazina	10 g de muestra se somete a agitación con 25 mL de agua ultrapura durante 2h. El sobrenadante se filtra a vacío y se repite el procedimiento juntando al final ambas porciones.	SPE con cartuchos de intercambio iónico MCX utilizando 3 mL de acetonitrilo con 4% hidróxido amónico como eluyente.	LC-DAD	*Papadopoulos et al., 2012*

Sedimento	Atrazina, Cianazina, DEA, Prometrina, Propazina, Terbutilazina	3 g de muestra, 12 mL (90:10 ACN/tampón fosfato, pH=7,5). 10 min de agitación + 6 min de centrifugación. Se retira el sobrenadante, y se repite la extracción.	---	LC-MS	*Dong et al., 2015*
Vegetales (raíces y partes verdes), suelos, pescados	Atrazina, Simazina	2 h de agitación. Suelo:100 g muestra + 100 mL de cloroformo Raíces vegetal: 15 g muestra + 50 mL acetona Hoja vegetal: 5 g muestra + 30 mL acetona Músculo pescado: 50 g + 50 mL de metanol.	SPE, empleando C_{18} como adsorbente y 3mL de acetato de etilo como eluyente.	HPLC-DAD	*Baranowska et al., 2008*
Pescados, crustáceos	Simazina, Simetrina	5 g de muestra + 5 g de sulfato sódico anhidro: agitación (2x30 mL de ACN). Se lleva a sequedad y se redisuelve en 10 mL de hexano.	SPE con 5 g de florisil + 1 g de sulfato sódico anhidro. Elución con mezcla de acetona:hexano (10:90, v/v).	GC-MS	*Tsuda et al., 2009*
Ultrasonidos (US)					
Sedimento	Atrazina, Propazina, Simazina, Terbutilazina	1 g de sedimento + 30 mL de hexano:acetona (1:1, v/v) se deja en baño de ultrasonidos durante 1h. Se centrifuga 5 min a 2500 rpm.	SPE, con 2 g de alúmina como adsorbente, y elución secuencial con 20 mL de la mezcla de acetona:hexano (2:1, v/v) seguida de 20 mL de la misma mezcla en proporción 1:10 (v/v).	GC-MS	*Villaverde et al., 2008*
Sedimento	Atrazina, DEA, DIA, Terbutilazina	Extracción con una mezcla de metanol:acetato de etilo (15:10, v/v) durante 30 min.	---	HPLC-MS/MS	*Nödler et al., 2013*
Cereales	Atrazina, Prometrina, Simazina	2 g de muestra + 40 mL de MeOH en baño de ultrasonidos 90 minutos.	---	HPLC-MS	*You et al., 2007*
Patatas	Ametrina, Atrazina, Prometrina, Propazina, Simazina, Terbutrina	5 g de patatas + 15 mL de agua Milli-Q en baño de ultrasonidos (10 min), se añade una disolución de diclorometano:acetona:hexano (1:1:1, v/v/v) y se extrae en ultrasonidos (10 min). Se centrifuga (10 min) para separar las fases.	Se emplean cartuchos Oasis MCX como adsorbente, y la elución se realiza con un mezcla de 4 mL de hidróxido amónico:metanol (15:85, v/v).	HPLC-DAD	*Rodríguez-Gonzalo et al., 2009*

Pescados, sedimentos	Atrazina, Simazina	5 g de muestra, 15 min con 3 porciones de 30 mL de hexano:diclorometano (1:1, v/v).	SPE con florisil como adsorbente. Elución con 25 mL de mezcla n-hexano:acetato de etilo (14:1 v/v) y 25 mL de la misma mezcla en proporción 15:10 (v/v).	HPLC-UV	*Salvadó et al., 2006*
Almejas, algas	Atrazina, DEHA, DET, DIHA, HA, HT	5 g de muestra liofilizada y homogeneizada, extracción con ultrasonidos y agitación manual utilizando una mezcla de metanol:agua (4:1, v/v).	Cartuchos Oasis HLB. Elución con mezcla de metanol:acetona :acetato de etilo (2:2:1, v/v/v) con 0,1% de ácido fórmico.	LC-MS/MS	*Carafa et al., 2007*
Extracción asistida con energía de microondas (MAE)					
Sedimento	Terbutilazina	3 g de sedimento + 25 mL de una mezcla de acetona:hexano (1:1, v/v). Extracción a 60ºC, 1600 W y 100 psi durante 10 min.	---	UPLC-MS/MS	*Kalogridi et al., 2014b*
Cereales	Atrazina, Pometrina, Simazina	2 g de muestra + 20 mL de MeOH. Extracción a 105ºC, 600 W durante 10 min.	---	HPLC-MS	*You et al., 2007*
Cereales	Ametrina, Atrazina, Propazina, Simazina	1 g de muestra + 4 g de arena de cuarzo y metanol a 600 W de potencia.	---	LC-MS	*Wang et al., 2012*
Hígado de oveja	Atrazina Cianazina Prometrina Simazina	1 g muestra + 10 mL de MeOH, 70ºC, 6 min.	SPE con 3 g de óxido de aluminio colocado entre 2 capas de 1,5 g de sulfato sódico anhidro cada una como adsorbente, utilizando como eluyente 10 mL de MeOH.	HPLC-UV	*Cheng et al., 2007*
Leche en polvo	Ametrina, Atrazina, Prometrina, Propazina, Simetrina.	Se realiza la extracción de 1,5 g de muestra, durante 20 min a 102ºC utilizando 4,6 mL de extractante (metanol con 0,1% de agua a pH=12).	SPE con cartuchos MCX. Se acidifica el sobrenadante obtenido en la extracción con 1 mL de HCl. Se eluye con una disolución de hidróxido amónico al 4% en metanol.	LC-MS/MS	*Fang et al., 2012*
Mejillones, almejas, sedimento, berberechos	Siete triazinas (entre ellas Simetrina)	0,5 g de muestra, extracción con metanol.	Se utilizó una columna con 22 g de alúmina y una mezcla de acetato de etilo:hexano (1:4, v/v) como eluyente.	LC-MS	*Fernández et al., 2013*

Extracción con disolventes a presión (PLE)					
Sedimento	Atrazina, DEHA, DET, DIHA, HA, HT	5 g de muestra, extracción con una mezcla de metanol:agua (4:1, v/v), a 100ºC y 1500 psi. 3 ciclos.	Cartuchos Oasis HLB (200 mg) como adsorbente, y 5 mL de una mezcla de MeOH:acetona :acetato etilo (2:2:1) con 0,1% de ácido fórmico.	LC-MS/MS	*Carafa et al., 2007*
Sedimento	Atrazina, Cianazina, DEA, Simazina, Terbutilazina	Se realiza la extracción utilizando una mezcla de hexano:acetona (1:1 v/v), con Tª inicial de 45ºC y un aumento progresivo de 35ºC/min hasta los 180ºC.	SPE, con cartuchos de florisil.	GC-MS	*Devault et al., 2010*
Sedimento	Atrazina, Cianazina, DEA, DIA, Simazina, Terbutilazina	5 g de muestra con una mezcla de metanol:acetona (1:1, v/v) a 50ºC, 1500 psi.	SPE con cartuchos de 1 g de carbograph como adsorbente, utilizando 1 mL de MeOH + 8 mL de DCM :MeOH (95 :5, v/v) como eluyente.	LC-MS/MS	*Ricart et al., 2010*
Sedimento	Ametrina, Atrazina, DEA, DIA, Propazina, Simazina, Simetrina, Terbutilazina	5 g de muestra con una mezcla de metanol:acetona (50:50, v/v) a 80ºC, 1000 psi, 1 ciclo de 10 min.	---	GC-MS/MS	*Camino-Sánchez et al., 2011*
Alimentos vegetales	Ametrina, Atrazina, Prometrina, Propazina, Simazina, Terbutilazina	7 g de muestra con una mezcla de diclorometano:hexano (1:4, v/v), a 1000 psi, 2 ciclos (21 min cada uno).	SPE con cartuchos Oasis MCX. Elución con 4 mL de una mezcla de disolución de amonio al 25% y MeOH (15:85, v/v).	HPLC-UV	*Carabias-Martínez et al., 2007*
Almejas	Atrazina, Simazina	0,4 g de muestra se extraen con una mezcla de diclorometano:hexano (1:1, v/v) a 150ºC, 1500 psi, 2 ciclos de 10 min.	---	GC-MS	*Damásio et al., 2010*
Ostras y mejillones	Atrazina, Cianazina, DEA, DIA, Simazina, Terbutilazina	0,25 g de muestra liofilizada se extraen con una mezcla de MeOH:DCM (1:1,v/v) durante 5 min a 100ºC y 200 psi.	SPE, con 5g de florisil como adsorbente.	GC-MS	*Köck et al., 2010*

En los últimos años las tendencias para simplificar los procedimientos analíticos han derivado en el desarrollo de nuevas metodologías que permiten la determinación de contaminantes en matrices complejas con una serie de ventajas tales como: reducción de las etapas de purificación y concentración, minimización del uso de disolventes orgánicos, especialmente los halogenados, y mejora en los límites de detección. En este contexto, técnicas como microextracción en fase sólida (SPME), extracción mediante barras agitadoras (SBSE), dispersión de la matriz en fase sólida (MSPD) y "*Quick, Easy, Cheap, Effective, Rugged and Safe*" (QuEChERS), son apropiadas y todas ellas han sido aplicadas respectivamente, al análisis de pesticidas en muestras de vegetación *(Walorczyk, 2008; Hu et al., 2010; Barchanska et al., 2014; Lee et al., 2016),* pescados y mariscos *(Lacroix et al., 2014; Dehaut et al., 2016; Rombaldi et al., 2015; Masiá et al., 2013)* y sedimentos *(Xu et al., 2016; Camino-Sánchez et al., 2011; Rocha et al., 2015; Brondi et al., 2011).*

En este trabajo, la técnica seleccionada para la extracción de triazinas en matrices sólidas es la MSPD debido a que es una alternativa interesante en la preparación de muestra, ya que supera muchos inconvenientes de los métodos tradicionales. Esta técnica se caracteriza por su flexibilidad, selectividad, la posibilidad de realizar la extracción y limpieza en un único paso, el bajo consumo de disolvente y un pretratamiento rápido de la muestra.

En la mayoría de los casos la MSPD da resultados iguales o mejores que los métodos clásicos; además, los métodos microanalíticos como MSPD, que permiten la reducción de los grandes volúmenes de disolventes orgánicos necesarios en una extracción sólido-líquido, son una importante contribución a la protección del medio ambiente (Química Verde) y del personal que realiza los análisis.

7.2.1. Dispersión de la matriz en fase sólida

La dispersión de la matriz en fase sólida es una técnica de extracción y purificación desarrollada para los métodos de análisis multi-residuo por Barker en 1989. Esta técnica permite la extracción de pesticidas en muestras dispersadas de forma homogénea en un soporte sólido, que normalmente es uno de los adsorbentes empleados con frecuencia para SPE. La matriz homogeneizada se introduce en una columna, se compacta, y a continuación los analitos son aislados de la matriz por elución con disolventes orgánicos. La principal ventaja de esta técnica es que permite llevar a cabo varios pasos en la preparación de la muestra de manera simultánea, reduciendo de esta manera el tiempo de análisis y disminuyendo el consumo de disolventes, ya que la extracción y purificación de la muestra se realizan en un único paso *(García-López et al., 2008; Capriotti et al., 2013).*

La MSPD es una forma de cromatografía que no sólo es diseñada para separar componentes de la muestra sino que, ante todo, los rompe y dispersa en la fase sólida, generando una nueva fase mixta con propiedades cromatográficas específicas. Los componentes de la muestra se distribuyen sobre la superficie de la fase sólida como una función de interacciones con el soporte y los propios componentes de la matriz, convirtiéndose así, junto con el adsorbente, en el nuevo material de empaquetado de la columna.

El carácter específico de la nueva fase adsorbente/matriz y el rango de interacciones permite el aislamiento de analitos de diferente polaridad al pasar un disolvente a través de la columna. El analito retenido puede ser fácil y completamente eluido empleando un pequeño volumen de disolvente orgánico o una secuencia de disolventes. La principal diferencia entre MSPD y SPE es que la muestra se dispersa por toda la columna y no sólo se retiene en los primeros milímetros.

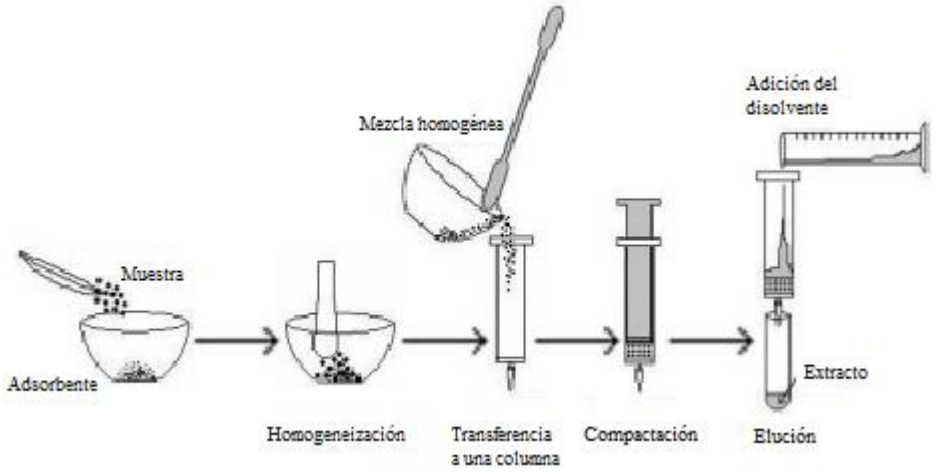

Figura 1.10. Esquema del procedimiento en MSPD

La muestra, que puede ser líquida, viscosa, semi-sólida o sólida, se coloca en un mortero que contiene el agente dispersante y se tritura hasta conseguir la completa dispersión de la muestra en el soporte *(Kristenson et al., 2006).* Este sistema actúa como un disolvente abrasivo que ayuda a la destrucción y ruptura de las membranas celulares de forma que la muestra se rompe en partes más pequeñas y sus componentes se distribuyen quedando dispersados en la superficie de dicha fase, de acuerdo con sus polaridades relativas.

La proporción entre las cantidades de muestra y de soporte sólido empleadas depende del tipo de muestra, pero normalmente oscila entre 1:1 y 1:4 *(Kristenson et al., 2006; Capriotti et al., 2013).* El mortero empleado conviene que sea de vidrio o ágata, ya que en los de porcelana u otro material poroso se ha demostrado que se producen pérdidas de analitos *(Barker, 2000).*

Tras mezclar bien en el mortero, se pasa a una columna con frita y se compacta. En el caso de muestras complejas, a menudo se coloca un adsorbente adicional, con propiedades diferentes a las del agente dispersante, en la parte inferior de la columna para mejorar el proceso de extracción. A continuación, se añade el eluyente y normalmente se deja que eluya a gravedad, aunque en algunos casos se puede realizar la elución aplicando presión o bien conectándolo a una bomba de vacío. Los analitos se eluyen con un pequeño volumen de un único disolvente o una secuencia de ellos empezando por el menos polar. Para la elución hay dos posibilidades: 1) los compuestos interferentes se eluyen directamente en un paso de lavado, mientras que los analitos son retenidos en la columna y se eluyen posteriormente con un disolvente diferente al de lavado; 2) los componentes de la matriz que interfieren son selectivamente retenidos en la columna y los analitos diana se eluyen directamente *(Kristenson et al., 2006; Capriotti et al., 2013).*

Los parámetros que afectan al proceso de extracción mediante MSPD son:

1- Naturaleza del agente dispersante. Tradicionalmente las fases más ampliamente utilizadas son materiales de fase reversa químicamente enlazados a un soporte sólido de sílica. En este tipo de adsorbentes, las partículas de sílice actúan como disgregantes ayudando a la ruptura de las membranas celulares, mientras que las cadenas alquílicas enlazadas solubilizan los componentes de la matriz sobre su superficie *(García-López et al., 2008).* Además, tienen la ventaja de contener grupos silanol no enlazados, tanto en la superficie de las partículas como en los poros, que interaccionan con el agua de la muestra actuando a su vez como agente desecante, lo que facilita la compactación y la elución.

Las fases enlazadas más empleadas son las de octilsilano (C_8) *(Kruve et al., 2008; Ramos et al., 2008; Ramos et al., 2009)* y octadecilsilano (C_{18}) *(Barker, 2007; Dantas-Silva et al., 2008; Moliner-Martínez et al., 2009; Sánchez-Brunete et al., 2010; Pérez et al., 2012; Souza-Caldas et al., 2013).* La fase C_8 tiene una cadena de menor longitud, siendo por ello más polar que C_{18}, por lo que se utiliza para extraer compuestos más polares, mientras que la fase C_{18} se emplea cuando se quieren extraer compuestos menos polares. En el caso de matrices vegetales ambas fases han sido empleadas de manera similar; sin embargo, el mayor tamaño de la cadena del adsorbente C_{18}, hace que éste sea más utilizado cuando se analizan matrices de origen animal, especialmente aquellas con mayor contenido lipídico. Con este tipo de material es posible obtener extractos libres de grasa de muestras de mariscos *(Tao et al., 2014; Rombaldi et al., 2015),* hepatopáncreas de cangrejo *(Souza-Caldas et al., 2013)* y pescados grasos *(Stanley et al., 2009; Tao et al., 2014).*

Otros materiales dispersantes empleados, aunque con menor frecuencia, son los materiales polares no enlazados, como sílica, florisil, o alúmina, los cuales interactúan con los componentes de la muestra exclusivamente por adsorción y no son capaces de disolverla. Las propiedades de adsorción de estos agentes se pueden ajustar en función de su contenido en agua y de su carácter ácido o básico; así, por ejemplo, la sílica ácida forma enlaces de hidrógeno entre sus grupos silanol con analitos que contengan grupos amino en su estructura. Debido a su alta polaridad, este tipo de materiales presenta una gran capacidad de adsorción de compuestos polares presentes en matrices no polares y han sido muy empleados en análisis medioambientales. Así, Lin et al. *(2010),* escogieron la alúmina como dispersante en un estudio de compuestos orgánicos nitrogenados en vegetales; Negreira et al. *(2013),* utilizaron florisil como adsorbente en el análisis de 8 filtros UV en muestras de pescados y crustáceos; y Chen et al. *(2014),* emplearon sílica para realizar extracción mediante MSPD de declorano en muestras de pescado.

Otro tipo de adsorbentes son los de naturaleza inerte, como la tierra de diatomeas, la Celita o la arena. Estos materiales se utilizan principalmente para permitir una rápida elución de las interferencias que no se logran retener por otros adsorbentes *(Kristenson et al., 2006; García-López et al., 2008; Capriotti et al., 2013).* El material inerte más comúnmente empleado como agente dispersante para extracción de distintos contaminantes es la tierra de diatomeas; así se ha empleado para extraer triclosanos y benzotriazoles en sedimentos *(González-Mariño et al., 2010; Carpinteiro et al., 2012),* Atrazina, DEA, DEDIA, DIA y HA en plantas *(Barchanska et al., 2014)* y fármacos de uso habitual en muestras de pescado *(Hertzog et al., 2015).*

Los materiales basados en carbón han sido menos empleados como dispersantes, aunque en los últimos años han cobrado interés. Estos materiales presentan una estructura no porosa y un gran área superficial permitiendo un rápido procesado de las muestras; además presentan una gran afinidad por compuestos orgánicos tanto polares como apolares. Uno de los adsorbentes basados en carbón más utilizados es el carbón negro grafitizado (GCB), el cual presenta gran afinidad por moléculas planas y retiene los pigmentos y esteroles presentes habitualmente en alimentos y productos naturales. Barriada-Pereira et al. *(2010),* estudiaron distintos agentes dispersantes para la extracción de pesticidas organoclorados en hígado de pescado, seleccionando carbón como adsorbente debido a la mayor eficiencia de extracción obtenida.

Otra clase de materiales son los polímeros de huella molecular (MIPs), que aunque son ampliamente utilizados en la técnica SPE, su empleo en MSPD es bastante reciente. Se han empleado para extraer cloranfenicol en muestras de suelo *(Wang et al.,*

2011b), imidacloprid en muestras de arroz *(Chen et al., 2012)* y para el análisis de triazinas en suelos, tomate y fresa *(Wen et al., 2012).*

Finalmente, en algunos trabajos también se han empleado mezclas de adsorbentes de distinta naturaleza. Así, por ejemplo, Cao et al. *(2015)* emplean como dispersante una mezcla de C_{18} con florisil a partes iguales para analizar dieciséis pesticidas en distintas plantas de té.

2- Uso de coadsorbentes. Como ya se dijo anteriormente, en los análisis de matrices complejas es habitual el uso de coadsorbentes de naturaleza distinta a la del agente dispersante, para obtener una mayor limpieza del extracto. A modo de ejemplo, en el caso de matrices vegetales cuando se utiliza C_8 o C_{18} como dispersante, se emplean coadsorbentes en fase normal como alúmina *(Abhilash et al., 2007)* y florisil *(Dantas-Silva et al., 2008; Moliner-Martínez et al., 2009; Fang et al., 2009; Sánchez-Brunete et al., 2010).* Otras combinaciones encontradas en la literatura de agente dispersante y coadsorbente respectivamente son, por ejemplo, alúmina y tierra de diatomeas *(Lin et al., 2010),* florisil con C_{18} *(Negreira et al., 2013),* tierra de diatomeas con sílica *(González-Mariño et al., 2010)* o sílica y florisil *(Chen et al., 2014).*

En los últimos años se ha incrementado el uso de cartuchos multicapa, donde cada capa cumple un papel específico en la eliminación de compuestos interferentes. Un tipo de cartuchos muy utilizado es el de doble capa formado por Envi-Carb y PSA, separados ambos adsorbentes por una frita *(Shimelis et al., 2007).* El adsorbente Envi-Carb posee gran afinidad por moléculas planas y puede eliminar pigmentos tales como clorofilas o carotenoides, y esteroles, presentes en productos naturales y en alimentos. El adsorbente PSA, consiste en una fase polimérica enlazada de etilendiamina-N-propil que contiene aminas primarias y secundarias, y presenta fuerte afinidad y gran capacidad para retener ácidos grasos, ácidos orgánicos, azúcares y algunos pigmentos polares. Otra combinación de coadsorbentes encontrada en la bibliografía es florisil y carbón, empleada por García-Rodríguez et al. *(2012)* en el análisis de diecisiete pesticidas en algas.

3- Eluyentes. La polaridad relativa del eluyente es determinante en cuanto a qué se consigue eluir y qué se queda en la columna, ya que su elección depende de la polaridad del analito. Para sustancias apolares se emplean disolventes apolares tales como hexano, diclorometano o una mezcla de ambos, mientras que cuando los analitos tienen polaridad media o alta es necesario utilizar disolventes polares. En el caso concreto de los herbicidas triazínicos, los eluyentes más empleados son diclorometano *(Tseng et al., 2004, Wen et al., 2012),* acetonitrilo *(Ferrer et al., 2005; Souza-Caldas et al., 2013),* acetato de etilo *(Ramos et al., 2009; Rombaldi et al., 2015)* y metanol *(Wen et al., 2012).* También se han empleado mezclas de varios disolventes como

acetonitrilo:metanol (9:1,v/v) *(Gaunt et Barker, 2000)* o metanol:acetato de etilo (2:1, v/v) *(Rocha et al., 2015).*

Cuando además de las triazinas también se analizan sus productos de degradación, en algunos casos puede ser necesario acidificar el eluyente para lograr una extracción más eficaz de los metabolitos de mayor polaridad; así, Barchanska et al. *(2014)* emplean una disolución de acetonitrilo acidificada con HCl 0,1M:metanol (50:50, v/v) como eluyente para la determinación de Atrazina y tres de sus metabolitos (DEA, DIA y HA) en plantas.

Como ya se mencionó anteriormente, antes de llevar a cabo la elución de los analitos de interés, puede realizarse una etapa previa de lavado con el fin de eliminar interferencias que puedan afectar posteriormente al análisis. Wen et al., *(2012)* analizan triazinas en distintas matrices mediante extracción MSPD empleando MIPs como dispersante y realizando un lavado previo con 5 mL de agua antes de proceder a la elución de las triazinas con un disolvente orgánico.

4- Naturaleza de la matriz. Los resultados obtenidos para un determinado analito variarán en función de la matriz empleada, del contenido en lípidos, proteínas, etc. y de su distribución en la matriz. A modo de ejemplo, en un estudio realizado por Ramos et al. *(2009),* donde se utilizó el mismo procedimiento de extracción de triazinas en distintos tipos de frutas, se observó que en el caso de las peras la extracción fue satisfactoria (%R > 91%), mientras que para uvas y manzanas, las recuperaciones fueron más bajas (%R < 71%). Wen et al. *(2012),* llevaron a cabo el análisis de cuatro triazinas (Ametrina, Atrazina, Propazina y Simazina en distintas matrices (suelo, fresa y tomate) confirmando también la influencia que tiene la matriz en la técnica MSPD, puesto que en las muestras de suelo y fresa los resultados fueron satisfactorios para todas las triazinas (%R > 70%) excepto Prometrina, mientras que en el caso del tomate los porcentajes de recuperación estuvieron en el rango 53-64%.

7.2.2. Aplicaciones de la Dispersión de la matriz en fase sólida

Debido a su simplicidad y elevado rendimiento, en los últimos años se han desarrollado numerosos métodos de MSPD para la extracción de residuos de pesticidas y otros contaminantes en muestras biológicas *(Beceiro-González et al., 2012).* Sin embargo, en el caso de las triazinas, las referencias son todavía muy escasas y en la mayoría de los estudios se incluyen pocas triazinas y casi ningún producto de degradación.

En lo que respecta a matrices de origen vegetal, se han analizado una gran variedad de contaminantes con esta técnica empleándose gran diversidad de agentes dispersantes. Utilizando C_{18} como adsorbente se han determinado micotoxinas en

muestras de alimentos vegetales *(Rubert et al., 2012),* diversos pesticidas en plantas medicinales *(Cheng et al., 2013)* y en el tallo de la palmera de coco *(de Jesús et Navickiene, 2014).* El florisil se ha empleado en un estudio de residuos de pesticidas también en el tallo de la palmera de coco *(Ferreira et al., 2013),* para analizar fenoxsulam en muestras de arroz y suelos *(Kaur et al., 2014)* y en un reciente trabajo sobre colorantes sintéticos en polvo de chile *(Chen et al., 2015).* Otro adsorbente muy empleado en matrices de origen vegetal es la sílica, utilizado para analizar pesticidas en plantas medicinales *(dos Santos et al., 2013),* OCPs y PCBs en aceites vegetales comestibles *(Li et al., 2014)* y en semillas de cacahuetes y soja *(Zhan et al., 2016),* y flavonoides en polen *(Ma et al., 2015).* Por otra parte, Lin et al. *(2010)* analizaron pesticidas nitrogenados eligiendo la alúmina como adsorbente y Zhou et al. *(2015)* utilizaron MIPs para determinar pesticidas en muestras de zanahoria y yacón.

En el caso de pescados y mariscos, la mayor parte de los estudios emplean C_{18} como agente dispersante. Con este adsorbente se han analizado PAHs en músculo de pescados *(Pensado et al., 2005);* PCBs y PAHs en tejido de renacuajo *(Stanley et al., 2009);* sulfonamidas en tejidos de pescados *(Lu et al., 2011)*; fenicoles en camarón y pescado *(Tao et al., 2014; Pan et al., 2015).* Otros adsorbentes empleados son de naturaleza polar; así Negreira et al. *(2013)* optimizaron las condiciones para la determinación de filtros UV en muestras de moluscos y pescados empleando florisil como agente dispersante y Chen et al. *(2014)* emplearon sílica para la determinación de declorano en pescados. En relación a los materiales inertes, también han sido empleados aunque con menor frecuencia; Fernández-González et al. *(2010)* escogieron como adsorbente la tierra de diatomeas para analizar PAHs en muestras de mejillón.

Como ya se comentó anteriormente, en lo que respecta a la determinación de herbicidas triazínicos, son muy pocas las aplicaciones de MSPD, y en la mayoría de ellas se incluyen muy pocas triazinas. La primera referencia recogida en la literatura solamente realiza la extracción de Simazina en caña de azúcar *(Tseng et al., 2004)* homogeneizando la muestra con adsorbente C_{18}, posterior limpieza con florisil y elución con diclorometano. Un año después, Ferrer et al. *(2005)* determinaron varios pesticidas en aceitunas, entre los que se encuentran Simazina y Terbutilazina, empleando Bondesil-NH_2 como adsorbente, florisil como coadsorbente y acetonitrilo como eluyente.

Los estudios posteriores incluyen mayor número de triazinas. Ramos et al. *(2008)* y Ramos et al. *(2009),* combinaron la técnica MSPD con extracción ultrasonidos para la extracción de nueve triazinas en frutas, empleando C_8 como adsorbente y acetato de etilo como disolvente de extracción. En el primer trabajo, se homogenizan 700 mg de muestra (manzana, pera y albaricoque) con 700 mg de adsorbente realizando la elución

con 700 µL de eluyente. En el trabajo publicado en 2009 se reduce la cantidad de muestra y de adsorbente a 500 mg y se aplica, además de a manzanas y peras, a uvas y naranjas. Aunque son los dos artículos de la bibliografía que determinan mayor número de triazinas empleando esta técnica de extracción, entre las que se encuentran siete de las nueve triazinas incluidas en este trabajo, es importante destacar el hecho de que no se determina ni Cianazina ni Simazina, siendo esta última uno de los herbicidas legislados, y por ello de gran importancia en los estudios medioambientales.

Más adelante Wen et al. *(2012)* aplicaron la técnica MSPD para el análisis de cuatro triazinas (Ametrina, Atrazina, Propazina y Simazina) en muestras de fresa, tomate y suelos, utilizando polímeros de impresión molecular (MIPs) como agente dispersante. En el caso de las matrices vegetales, se homogenizan 0,2 g de muestra con 0,6 g de MIPs y la mezcla se transfiere a una jeringa que contiene el coadsorbente C_{18} (0,05 g). A continuación se lava con 5 mL de H_2O realizándose posteriormente la elución con 5 mL de acetato de etilo en el caso de las fresas y 10 mL de diclorometano en el caso de los tomates.

Un año después, Radisic et al. *(2013),* realizaron el análisis de 15 pesticidas (entre ellos Atrazina, Propazina y Simazina) en cuatro tipos de frutas (albaricoques, manzanas, fresas y naranjas). El procedimiento consiste en homogeneizar la muestra con tierra de diatomeas, y posterior elución de los analitos con diclorometano.

Recientemente Barchanska et al. *(2014)* publicaron el primer trabajo en el que se utiliza extracción MSPD para determinar, además de Atrazina, tres de sus metabolitos (DEA, DIA y HA) en plantas. Se estudiaron dos adsorbentes, tierra de diatomeas y gel de sílice, obteniendo recuperaciones ligeramente más altas con tierra de diatomeas para las muestras de hojas y recuperaciones mayores con sílica en el caso de las raíces. De entre los disolventes estudiados para la etapa de elución (metanol, acetonitrilo, disoluciones de ambos disolventes acidificadas con HCl 0,1M, y otras combinaciones) se escogió una mezcla de disolución de acetonitrilo acidificada con HCl 0,1M:metanol (1:1, v/v) ya que, debido a la mayor polaridad de los productos de degradación derivados de la Atrazina, fue necesario acidificar el eluyente para lograr una mayor eficiencia de extracción de los mismos.

En el caso de matrices de origen animal, sólo se han encontrado tres artículos dedicados a la determinación de triazinas, los cuales han empleado C_{18} como agente dispersante. Gaunt et Barker *(2000),* realizaron un estudio para la determinación de tres triazinas (Atrazina, Cianazina y Simazina) en tejidos de pescados utilizando una mezcla (9:1, v/v) de ACN:MeOH como disolvente de elución. Souza-Caldas et al. *(2013)* utilizaron MSPD asistida por vórtex para extraer Atrazina y otros pesticidas en hígado de pescado y en hepatopáncreas de cangrejo, homogenizando el adsorbente con 0,2 g de

muestra en el caso del hígado y con 0,5 g de muestra en el caso de hepatopáncreas, empleando acetonitrilo como eluyente. Rombaldi et al. *(2015)*, analizaron una serie de contaminantes, entre los que se encuentra la Atrazina, en tejido de mejillón mediante MSPD asistida por agitación vórtex. El procedimiento consiste en homogeneizar la muestra con Na_2SO_4 y con concha de mejillón, que actúa como adsorbente, y posterior elución con de acetato de etilo.

Finalmente, en relación a matrices sólidas medioambientales, las aplicaciones de MSPD son mucho más escasas y recientes que para muestras biológicas. La mayoría de los procedimientos de la bibliografía utilizan dispersantes de fase reversa, especialmente C_{18} *(Sánchez-Brunete et al., 2010; Pérez et al., 2012)* y de naturaleza inerte como la tierra de diatomeas *(González-Mariño et al., 2010; Carpinteiro et al., 2012)*, empleando adicionalmente un adsorbente en fase normal para mejorar la limpieza del extracto. Así se ha empleado la combinación de tierra de diatomeas con sílica para la extracción de triclosanos *(González-Mariño et al., 2010)* y benzotriazoles *(Carpinteiro et al., 2012)* en sedimentos, y la combinación de C_{18} con florisil para la determinación de parabenos y alquilfenoles *(Pérez et al., 2012)* y triclosanos *(Sánchez-Brunete et al., 2010)* en suelos. Los materiales basados en carbón han sido menos empleados como dispersantes; sin embargo, estudios recientes han proporcionado resultados altamente satisfactorios, no siendo además necesario el uso de un coadsorbente adicional en la limpieza del extracto *(Li et al., 2014).*

En lo que respecta a herbicidas triazínicos, sólo se han encontrado dos referencias en la literatura. Como ya se comentó en referencia a tejidos de origen vegetal, Wen et al. *(2012)* aplicaron la técnica MSPD para el análisis de cuatro triazinas (Ametrina, Atrazina, Propazina y Simazina) en muestras de suelo, frutas y vegetales utilizando polímeros de impresión molecular como dispersante y C_{18} como coadsorbente. En el caso del suelo, los mejores resultados se obtuvieron mezclando 0,2 g de muestra con 0,2 g de MIPs realizando la elución con 5 mL de metanol.

Recientemente, Rocha et al. *(2015)* estudiaron dieciocho pesticidas (entre ellos Atrazina), en muestras de aguas, pescados y sedimentos. Para realizar la extracción de las muestras de aguas se utilizó la técnica SPE, para las de pescado QuEChERS, y en el caso de los sedimentos se realizó mediante MSPD, empleando C_{18} como adsorbente y MeOH:acetato de etilo (2:1, v/v) como eluyente.

8. MÉTODOS DE CONCENTRACIÓN

En los análisis de compuestos orgánicos en muestras medioambientales a niveles traza, es necesaria una etapa de concentración de la muestra antes de llevar a cabo la separación cromatográfica, ya que la concentración de los analitos suele estar por

debajo de los límites de detección y cuantificación de los equipos instrumentales *(Cámara-Rica et al., 2004).*

Una forma rápida de concentrar los eluatos es empleando un rotavapor, en el que el disolvente se elimina a presión reducida, lo que disminuye considerablemente su punto de ebullición. En la evaporación con rotavapor, el matraz está rotando mecánicamente en un baño a una temperatura adecuada, formándose una fina película del disolvente en una gran superficie facilitando su evaporación. Este método ha sido empleado en diversos estudios de triazinas y sus productos de degradación en aguas *(Huff et Foster, 2011),* vegetales *(Carabias-Martínez et al., 2007; You et al., 2007),* alimentos de origen animal *(Tsuda et al., 2009)* y sedimentos *(Dong et al., 2015).*

Otra técnica de concentración consiste en pasar un flujo suave de un gas inerte sobre la superficie del eluato. De esta forma se consigue desplazar el equilibrio líquido-vapor ya que se elimina continuamente el vapor generado. Este procedimiento es lento por lo que se utiliza para volúmenes de disolvente pequeños (inferiores a 25 mL). La evaporación depende de la velocidad del gas, la posición relativa de la conducción de gas respecto al disolvente, de la temperatura y del área superficial que ocupa el disolvente. El gas utilizado normalmente es el nitrógeno. Éste es el método de concentración más empleado en el análisis de triazinas y/o sus productos de degradación. Se ha empleado para concentrar extractos de aguas *(Lissalde et al., 2011; Kalogridi et al., 2014a),* vegetales *(Baranowska et al., 2008; Rodríguez-Gonzalo et al., 2009),* alimentos de origen animal *(Köck et al., 2010; Fang et al., 2012)* y sedimentos *(Papadopoulos et al., 2012; Rocha et al., 2015).*

En el caso de analitos orgánicos volátiles, es importante no llevar la evaporación del disolvente a sequedad en el rotavapor, ya que se pueden producir pérdidas de los compuestos de interés por su descomposición o volatilización. Si es necesario llevar el extracto a sequedad, debe hacerse sin calentamiento, combinando la técnica de rotavapor con un burbujeo suave de un gas inerte *(Cámara-Rica, 2004; Iglesias-García et al., 2008).* Diversos autores han empleado esta técnica combinada para realizar análisis de triazinas en muestras de origen vegetal *(Chen et al., 2012)* y animal *(Lin et al., 2010),* y en sedimentos *(Villaverde et al., 2008).*

9. TÉCNICAS DE DETERMINACIÓN

Una vez extraídos los analitos de la matriz debe llevarse a cabo el análisis de los mismos mediante la técnica adecuada. Las técnicas cromatográficas de alta resolución tanto de gases (GC) como de líquidos (LC), son las más utilizadas para la determinación de herbicidas triazínicos y sus productos de degradación.

9.1. Cromatografía de gases

En lo que a cromatografía de gases se refiere, existen diferentes estudios que emplean esta técnica acoplada con espectrometría de masas (GC-MS) para el análisis de triazinas en muestras de diferentes matrices ambientales utilizando distintos métodos de extracción.

Las columnas capilares más empledas en GC son las columnas recubiertas de fases apolares a base de polisiloxanos con radicales orgánicos, que proporcionan mayor o menor polaridad a la fase estacionaria en función de su naturaleza. Las fases estacionarias más utilizadas en el análisis de triazinas son 5% fenil-95% metilpolisiloxano *(Shen et al., 2002),* 5% fenil-95% dimetilpolisiloxano *(Berzas-Nevado et al., 2007; Djozan et Ebrahimi, 2008)* y 100% dimetilpolisiloxano *(Djozan et Ebrahimi, 2008; Djozan et al., 2010),* con longitudes de columna que varían entre los 30 y 50 m según el estudio.

Diferentes autores han escogido esta técnica de determinación para el análisis de triazinas en muestras acuosas utilizando como métodos de extracción SBSE *(León et al., 2003),* DLLME *(Nagaraju et Huang, 2007)* ó SPE *(Navarro et al., 2010; Portolés et al., 2011).*

También se han analizado triazinas en otro tipo de matrices ambientales mediante análisis con GC-MS. Hildebrant et al. *(2007)* realizaron un estudio sobre pesticidas prioritarios y sus productos de degradación (entre los que se encuentran Atrazina, DEA, Propazina, Simazina y Terbutilazina), en aguas subterráneas y en suelos. La extracción en muestras acuosas se llevó a cabo utilizando SPE, y en las muestras sólidas los compuestos fueron extraídos mediante PLE. En otro estudio de muestras de suelo, se empleó GC-MS y un método de microextracción en fase sólida para la determinación simultánea de 20 pesticidas pertenecientes a distintos grupos (entre ellos Atrazina, Prometrina y Simazina) *(Durović et al., 2010).*

Por otra parte, se han utilizado diferentes técnicas de extracción y GC-MS para la determinación de residuos de pesticidas en sedimentos. Villaverde et al. *(2008)* desarrollaron un método multi-residuo para la determinación de 28 pesticidas prioritarios (entre ellos Atrazina, Propazina, Simazina, y Terbutilazina) en sedimentos de ríos comparando tres métodos de extracción: US, PLE y extracción con Soxhlet seguida de SPE. Todas las técnicas de extracción obtuvieron recuperaciones aceptables para las triazinas.

Camino-Sánchez et al. *(2013)* validaron un método para la determinación simultánea de 86 contaminantes orgánicos persistentes en sedimentos marinos mediante PLE seguida de purificación con SBSE. Entre los contaminantes estudiados cabe

destacar el estudio de las siguientes triazinas y metabolitos: Ametrina, Atrazina, DEA, DIA, Prometrina, Propazina y Terbutilazina. También se ha empleado la extracción mediante la técnica QuEChERS y GC-MS para la determinación de cuatro pesticidas (entre ellos Atrazina) en sedimentos *(Brondi et al., 2011).*

Otras matrices en las que se han analizado triazinas empleando GC-MS son carne, fruta y verduras. En un trabajo desarrollado por Rosenblum et al. *(2001),* se llevó a cabo la determinación de pesticidas (entre ellos Atrazina y Simazina) en dichas matrices, mediante GC-MS y extracción Soxhlet.

Aunque apenas se han realizado estudios sobre triazinas en pescados y mariscos mediante esta técnica de determinación, Tsuda et al. *(2009)* emplearon esta técnica para analizar 29 pesticidas (entre ellos Simazina y Simetrina) utilizando agitación mecánica seguida de SPE para la extracción de los compuestos en músculos de pescado, mariscos y aguas. Recientemente, se ha desarrollado un método basado en extracción MSPD seguido de determinación por GC-MS para pesticidas (Atrazina entre ellos) en hígado de pescado y en hepatopáncreas de cangrejo *(Souza-Caldas et al., 2013).*

Cabe destacar el desarrollo que ha tenido el acoplamiento GC-MS, con la introducción de modernos detectores basados en cuadrupolos, los cuales resultan muy sensibles y aportan información estructural de la molécula, convirtiendo esta técnica en una herramienta muy fiable para la confirmación de muestras positivas *(Berzas-Nevado et al., 2007; Djozan et al., 2008; Djozan et al., 2010).* Pero aunque se han desarrollado métodos de análisis multi-residuo basados en cromatografía de gases acoplados a detectores de espectrometría de masas para determinar diferentes tipos de pesticidas en matrices ambientales (anilinas, organoclorados, organofosforados, piretroides, triazinas, triazoles…), la cromatografía de gases no está considerada como un método apropiado para el análisis de ciertos compuestos con elevado peso molecular y que contienen cadenas laterales polares, pues su baja volatilidad hace que sea necesario realizar una etapa de derivatización.

Esta técnica de determinación tampoco es aconsejable cuando los compuestos que se quieren determinar no son térmicamente estables o son compuestos que se encuentran en forma iónica puesto que en general son poco volátiles.

9.2. Cromatografía de líquidos

En los últimos años, el uso de cromatografía de líquidos se ha visto incrementado debido a una mayor sensibilidad de los detectores y a las limitaciones que, como ya se ha citado en el apartado anterior, presenta la cromatografía de gases siendo su principal limitación la aplicación al análisis de productos de degradación

hidroxilados ya que, debido a su polaridad, no son muy volátiles. Por ello, en este trabajo se emplea la cromatografía de líquidos (LC).

La separación cromatográfica se lleva a cabo poniendo en contacto dos fases inmiscibles entre sí. La muestra atraviesa la columna cromatográfica a través de la fase estacionaria mediante el bombeo de la fase móvil a alta presión, de manera que las especies de la muestra experimentan interacciones repetidas entre ambas fases. Los analitos de la muestra quedan retenidos diferencialmente en la fase estacionaria dependiendo de las interacciones con dicha fase. El grado de retención depende de la naturaleza del compuesto y de la composición tanto de la fase estacionaria como de la de la fase móvil. Los analitos emergen en orden creciente de interacción con la fase estacionaria, de manera que el más afín a la fase móvil emerge primero, mientras que el más retenido por la fase estacionaria es eluido en último lugar. Finalmente, los analitos llegan al detector que generará una señal proporcional a la concentración. La base de la separación cromatográfica será la diferencia de velocidad de migración entre los distintos analitos, para lo que deben optimizarse las condiciones experimentales y escoger adecuadamente las fases empleadas *(García-de Marina Bayo et Yusá-Marco, 2008; Gismera et al., 2009; Dean, 2014).*

En función del mecanismo de separación, se distinguen distintos tipos de cromatografía *(Loro-Ferrer et al., 2001; García-de Marina Bayo et Yusá-Marco, 2008; Gismera et al., 2009):*

- *Cromatografía de adsorción:* los componentes de la muestra son retenidos mediante adsorción selectiva en la superficie de un sólido de elevada superficie específica que constituye la fase estacionaria. Las moléculas de soluto y de la fase móvil compiten por las posiciones en el adsorbente.
- *Cromatografía reparto:* la fase estacionaria es un líquido que se encuentra impregnando un soporte sólido. El soluto se reparte entre la fase estacionaria líquida y la fase móvil de acuerdo con su solibilidad en cada una de ellas. Cuando la fase móvil es más polar que la fase estacionaria se realiza una cromatografía en fase reversa, y cuando la fase móvil es la más apolar se trabaja en fase normal. Las fases estacionarias más empleadas son fases enlazadas que suelen estar formadas por partículas de sílice, resistentes, porosas y con un diámetro de unos 3-5 µm**.** Su superficie está formada por grupos silanol químicamente reactivos. Cuando la reacción se produce con un grupo alquilo de mayor tamaño, los efectos estéricos impiden que todos los grupos silanol reaccionen, afectando al resultado de la separación. Para reducir este efecto, los grupos silanol libres se desactivan haciéndolos reaccionar con clorotrimetilsilano que, debido a su pequeño tamaño, puede unirse químicamente a muchos de ellos.

- *Cromatografía de intercambio iónico:* la fase estacionaria es un sólido que contiene grupos cargados positiva o negativamente, teniendo la capacidad de separar especies iónicas. Los solutos de carga opuesta a la fase estacionaria son atraídos por ésta mediante fuerzas electrostáticas.
- *Cromatografía de exclusión molecular*: en este tipo de separación cromatográfica, también llamada de gel permeación o de filtración, la fase estacionaria está constituida por un polímero entrecruzado de tamaño de poro definido. Esta modalidad cromatográfica es útil para especies neutras de alto peso molecular realizándose la separación en función de su tamaño.
- *Cromatografía de afinidad*: permite la separación de mezclas por su interacción específica con un determinado ligando de actividad: enzima-sustrato, antígeno-anticuerpo.

Las triazinas y sus productos de degradación poseen propiedades ácido-base y son relativamente polares, por lo que su separación mediante LC debe realizarse en fase reversa, con fase móvil de polaridad elevada y fase estacionaria apolar. Como fase estacionaria se suelen utilizar columnas recubiertas de fases no polares como alquil-sílicas: octadecil (C_{18}) *(Beale et al., 2010; Köck et al., 2010; Postigo et al., 2010; Huff et Foster, 2011; Lissalde et al., 2011; Baranowska et al., 2012; Köck-Schulmeyer et al., 2012; Huff et Foster, 2013; Hurtado-Sánchez et al., 2013; Li et al., 2013; Amaral et al., 2014; Homazava et al., 2014; Rocha et al., 2015; Rodrigues et al., 2016)* y octil (C_8) *(Papadopoulos et al., 2007; Beale et al., 2009; Beale et al., 2010),* donde los grupos alquilo están enlazados covalentemente a grupos silanol en la superficie de la sílica. Cuando la muestra pasa a través de este tipo de fase estacionaria, los analitos orgánicos interaccionan con la fase (fuerzas de Van der Waals e interacciones hidrofóbicas) quedando retenidos en ella.

En cuanto a las dimensiones de la columna, las longitudes más empleadas son 50 mm *(Hurtado- Sánchez et al., 2013; Homazava et al., 2014),* 100 mm *(Kalogridi et al., 2014a; Kalogridi et al., 2014b),* 125 mm *(Köck et al., 2010; Postigo et al., 2010; Köck-Schulmeyer et al., 2012),* 150 mm *(Huff et Foster, 2011; Rodrigues et al., 2016)* y 250 mm *(Beale et al., 2009; Beale et al., 2010)* y los diámetros de partícula más utilizados para las columnas convencionales varían entre 2,7 y 5 µm *(Beale et al., 2010; Köck et al., 2010; Postigo et al., 2010; Köck-Schulmeyer et al., 2012*; *Hurtado-Sánchez et al., 2013).*

El reciente desarrollo de columnas para cromatografía de líquidos de ultra-resolución (UPLC), que utilizan nuevas fases estacionarias con tamaño de partícula < 1,8 µm frente al tamaño empleado en columnas convencionales (2,7-5 µm), permite acortar significativamente el tiempo de análisis *(Montoro et al., 2007; Drozdzynski et*

al., 2008; Gervais et al., 2008; Brix et al., 2009; *Hurtado-Sánchez et al., 2013; Homazava et al, 2014; Kalogridi et al., 2014a; Kalogridi et al., 2014b).*

La cromatografía de líquidos de ultra-resolución presenta excelentes resultados, ya que es altamente robusta, fiable y reproducible. El fundamento cromatográfico es el mismo que el de HPLC, pero el uso de caudales más altos y un aumento del rendimiento, permite obtener mejor resolución de los picos cromatográficos, incrementar la sensibilidad y reducir de manera significativa el tiempo de análisis *(www.waters.com).*

Las columnas elegidas para la mayoría de las separaciones son columnas con relleno de C_{18} o C_8 que presentan puentes híbridos de etileno (BEH), debido a que ofrecen un intervalo de pH de operación más amplio. La presencia de ligandos trifuncionales produce una excelente estabilidad a bajo pH y una baja degradación de la columna. Esta estabilidad a pH bajo se combina con la estabilidad a pH alto de la partícula BEH *(www.waters.com).* Así, el empleo de UPLC con este tipo de columnas combina la mejora en prestaciones de las columnas rellenas de material híbrido con la capacidad del sistema de suministrar la fase móvil a alta presión, produciendo como resultado picos más estrechos y un aumento del rendimiento, la resistencia y eficacia. Además, se reduce el consumo de eluyente *(Gervais et al., 2008; Homazava et al., 2014; Kalogridi et al., 2014a; Kalogridi et al., 2014b; Rodrigues et al., 2016).*

El principal criterio a la hora de elegir una fase móvil es su compatibilidad con el sistema de detección. La selección del disolvente empleado como fase móvil, depende de dos factores fundamentales: las propiedades físico-químicas del disolvente y los efectos que estas propiedades tienen en el proceso cromatográfico. Hay que tener en cuenta que la separación cromatográfica depende de la distribución del analito entre la fase móvil y la fase estacionaria, que va a variar en función de su carácter hidrófilo o hidrófobo. Las triazinas presentan ambos comportamientos, ya que el anillo es hidrófilo y las cadenas aminoalquílicas presentes en las posiciones 4 y 6 son hidrófobas; por ello, como ya se dijo anteriormente, en el análisis de triazinas y de sus productos de degradación, se emplean fases móviles en fase reversa. Las fases móviles más empleadas en la literatura son mezclas de ACN:H_2O *(Brix et al., 2009; Mazzella et al., 2009; Beale et al., 2010; Köck et al., 2010; Postigo et al., 2010; Köck-Schulmeyer et al., 2012; Amaral et al., 2014),* MeOH:H_2O *(Cheng et al., 2007; Baranowska et al., 2008)* y ACN:H_2O:MeOH *(Beale et al., 2009).*

También es importante tener en cuenta el pKa tanto de las triazinas como de sus productos de degradación, pues estos últimos dependen fuertemente del pH. Por este motivo puede ser necesario utilizar un tampón o un modificador en la fase móvil para mejorar la separación. En el análisis de triazinas y sus productos de degradación se han

empleado pequeños volúmenes de ácidos débiles como ácido acético *(Carafa et al., 2007; Ferrer et Thurman, 2007; Huff et Foster, 2011; Barchanska et al., 2012; Barchanska et al., 2014; Zhang et al., 2014; Rocha et al., 2015)*, ácido fórmico *(Curini et al., 2000; Gervais et al., 2008; Hurtado-Sánchez et al., 2013; Homazava et al., 2014 Kalogridi et al., 2014a; Kalogridi et al., 2014b); y* sales como el acetato amónico *(Carabias-Martínez et al., 2007; Papadopoulos et al., 2007; Portugal et al., 2008; Smith et al., 2008; dos Santos et al., 2009; Lissalde et al., 2011; Hurtado-Sánchez et al., 2013; Rodrigues et al., 2016).* Estos modificadores o tampones se emplean en una o en ambas fases móviles.

9.3. Detectores

Respecto a los detectores más utilizados en cromatografía de líquidos para el análisis de triazinas se encuentran el detector ultravioleta (UV), el detector de red de diodos (DAD) y especialmente el detector de espectrometría de masas (MS), particularmente en tándem (MS/MS) por su elevada sensibilidad y selectividad *(LeBaron et al., 2008)*. En el presente trabajo, se han empleado DAD y MS/MS como detectores.

9.3.1. Detectores espectofotométricos

Los detectores espectrofotométricos (UV y DAD), están basados en la absorción de la radiación UV-visible por las moléculas de soluto. Aunque hay un gran número de sustancias que absorben esta radiación, como la mayoría de las moléculas orgánicas, estos detectores no tienen aplicación universal. Los detectores más sencillos son los de longitud de onda fija (detectores UV), que funcionan con radiación de una única longitud de onda generada por una lámpara específica. Gran parte de estos detectores operan a 254 nm empleando una lámpara de mercurio, lo que hace necesario el empleo de filtros *(Loro-Ferrer et al., 2001; Gismera et al., 2009).*

Debido a su baja selectividad, se han sustituido estos detectores por otros de longitud de onda variable, que permiten seleccionar la longitud de onda a la que los compuestos de interés presentan la máxima absorbancia. Aunque en los últimos años cada vez son menos utilizados, todavía hay autores que emplean este tipo de detectores para la determinación de pesticidas. Así, Dragus et al. *(2012)*, realizaron un análisis de Atrazina, Propazina y Simazina para estudiar la contaminación del agua, y Amaral et al. *(2014)*, propusieron el análisis de Atrazina, DEA y DIA en muestras acuosas naturales y minerales, escogiendo el detector UV.

Actualmente, la mayoría de los detectores de absorbancia son de red de diodos, que tiene la capacidad de registrar simultáneamente la señal a diferentes longitudes de onda, suministrando el espectro de la disolución que atraviesa la célula de detección, lo

que permite extraer el espectro UV de cada compuesto. Por ello, la posibilidad de la doble identificación, por tiempo de retención y por espectro UV, permite la confirmación de la existencia de un compuesto con mayor rigor *(Loro-Ferrer et al., 2001; Gismera et al., 2009).*

En la bibliografía, se encuentran numerosos trabajos para la determinación de triazinas y alguno de sus productos de degradación en matrices acuosas *(Zhao et al., 2011; Akdogan et al., 2013; Wu et al., 2013; Moliner-Martínez et al., 2015),* en sedimentos *(Silva et al.; 2010; Wu et al., 2010*; *Amadori et al., 2013),* en plantas *(Amelin et al., 2012; Barchanska et al.,2014)* y en muestras de origen animal *(Gaunt et Barker, 2000; Reindl et al., 2015),* en los que se emplea DAD como detector.

9.3.2. Detectores de espectrometría de masas

A pesar del uso tan extendido del detector DAD, hay que tener en cuenta que el rango de herbicidas que se aplican en los cultivos es muy amplio y, por ello, es importante disponer de un detector útil para para el análisis de diferentes clases de pesticidas simultáneamente utilizando un método analítico multi-residual. Para llevar a cabo este tipo de análisis multirresiduo, el detector más adecuado es el espectrómetro de masas.

Un espectrómetro de masas se compone de tres partes fundamentales: fuente de ionización, analizador de masas y detector *(Gismera et al., 2009).*

La fuente de ionización es la parte del espectrómetro donde se ionizan las moléculas de analito tras eliminar la fase móvil para poder llevar a cabo su posterior determinación en el espectrofotómetro de masas. Existen diferentes tipos de fuentes de ionización: bombardeo de átomos a gran velocidad (FAB), desorción de la matriz asistida por láser (MALDI), espectrometría de masas de iones secundarios (SIMS) e ionización a presión atmosférica (API) *(Niessen, 2007).*

Las fuentes API producen la ionización suave de un gran número de compuestos, lo que hace que no se originen demasiadas fragmentaciones del analito durante el proceso de ionización, siendo por ello las más utilizadas en LC-MS. Para lograr la formación de los iones, se aplica un potencial y una elevación de la temperatura. El voltaje aplicado puede ser positivo o negativo, dando lugar a dos modos de ionización, ionización en modo positivo (+) o ionización en modo negativo (-). El modo de ionización empleado depende de la afinidad protónica de los compuestos a determinar. Los compuestos que tienen grupos básicos con gran afinidad protónica que tienden a cargarse positivamente, se ionizarán en modo positivo, mientras que los compuestos con grupos ácidos y baja afinidad protónica que tienden a cargarse

negativamente, serán ionizados en modo negativo *(Loro-Ferrer et al., 2001; Niessen, 2007; Gismera et al., 2009).*

Dentro de las técnicas de ionización a presión atmosférica, se pueden diferenciar tres tipos: ionización química a presión atmosférica (APCI), fotoionización a presión atmosférica (APPI) e ionización con electrospray (ESI), siendo esta última la más versátil, ya que se puede aplicar tanto a analitos de polaridad media-baja como a analitos de polaridad elevada y gran peso molecular *(Loro-Ferrer et al., 2001; Niessen, 2007; Gismera et al., 2009).* Dado que las triazinas son moléculas relativamente polares y débilmente básicas, tienden a captar un protón cargándose positivamente, por lo que su ionización se suele llevar a cabo en modo positivo ESI (+).

La ionización por electrospray se basa en aplicar un campo eléctrico elevado para convertir la muestra líquida en un fino aerosol, que posteriormente dará lugar a la formación de iones en fase gas tras la evaporación de la fase móvil. Es la técnica de ionización a presión atmosférica más sensible, lo que permite utilizar flujos menores de gas. Su principal desventaja es la posibilidad de tener efecto matriz, dado que la ionización tiene lugar en fase líquida *(Niessen, 2007; Gismera et al., 2009)* (**figura 1.11**).

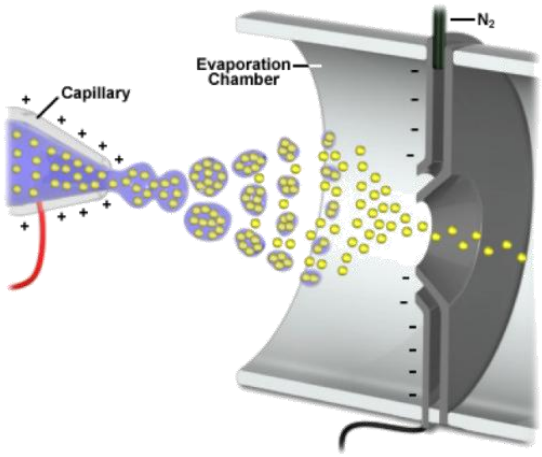

Figura 1.11. Fuente de ionización con electrospray

El analizador de masas lleva a cabo la separación de los iones obtenidos en la ionización en función de su relación masa/carga (*m/z*) *(Niessen, 2007; Gismera et al., 2009).* Existen distintos analizadores, y su elección depende del rango de masas que es capaz de analizar, la resolución, la sensibilidad y el coste.

Los analizadores de masas más utilizados en LC-MS y LC-MS/MS son la trampa de iones quadrupolares (IT), la trampa de iones lineales (LIT), el analizador de tiempo de vuelo (TOF), el cuadrupolo (Q), el triple quadrupolo (QqQ) y el *Orbitrap.* Uno de los detectores más utilizados es el de triple cuadrupolo, debido a su elevada

sensibilidad, selectividad y coste moderado. Además, permite trabajar em MS y en MS/MS *(Niessen, 2007; Gismera et al., 2009).* El QqQ ha sido el detector empleado para desarrollar los métodos de LC-MS/MS y UPLC-MS en esta Tesis.

Un cuadrupolo está compuesto por cuatro rodillos circulares organizados paralelamente como dos grupos de dos rodillos conectados eléctricamente entre sí, situándose los polos en torno a un eje central (**figura 1.12**). Mediante la aplicación de un campo eléctrico y un campo magnético los iones entran en el cuadrupolo y comienzan a oscilar, describiendo una trayectoria en función de su relación m/z.

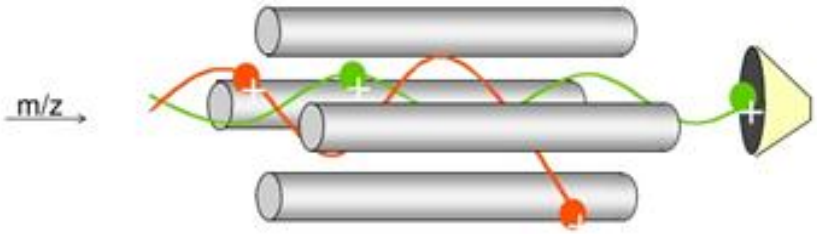

Figura 1.12. Cuadrupolo

La combinación de un campo eléctrico y una radiofrecuencia determinada permite la separación de los iones según la relación m/z, llegando al detector los iones seleccionados, mientras que el resto se desvían actuando el cuadrupolo como un filtro *(Niessen, 2007; Gismera et al., 2009).*

El triple cuadrupolo (QqQ), está formado por tres cuadrupolos colocados en serie, donde "Q" se refiere a los cuadrupolos donde se realiza la separación de los iones y "q" es la celda de colisión. (**Figura 1.13**). Una vez que los iones han pasado el primer filtro (Q_1), los iones seleccionados se fragmentan en la celda de colisión (q_2) en nuevos iones aplicando la energía de colisión adecuada y con ayuda del gas de colisión. Estos iones se dirigen hasta el tercer cuadrupolo (Q_3) y de éste al detector.

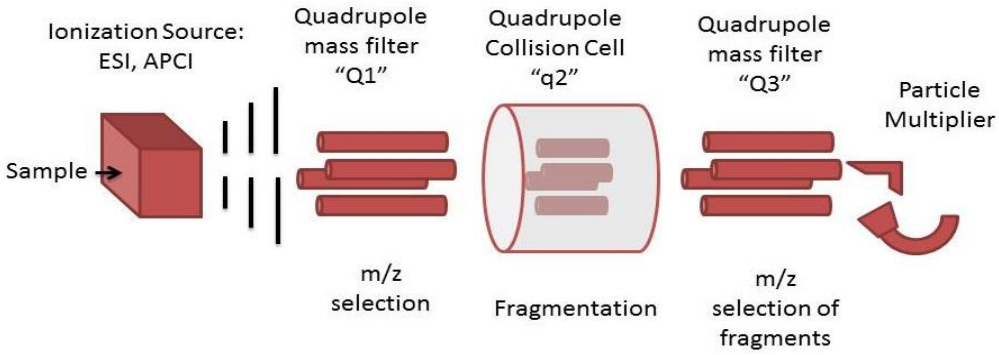

Figura 1.13. Esquema de un analizador de masas de triple cuadrupolo (QqQ)

Se utilizan distintos modos de trabajo, el barrido de iones y la adquisición de iones. En el modo de barrido se distingue entre:

-*Barrido total de iones (Full Scan),* en el que se obtiene un espectro de masas continúo en un rango definido. Todas las moléculas ionizadas llegan al detector y tiene lugar en el Q_1.

-*Barrido de iones producto (Product Ion Scan),* en el cual en el Q_1 se selecciona un ion (ion precursor) que se fragmenta en q_2 y se realiza el barrido de todos los fragmentos del ion precursor en el Q_3.

-*Barrido de iones precursor (Precursor Ion Scan),* en el que se lleva a cabo un barrido de todos los iones en el Q_1, se fragmentan en q_2 y se selecciona una única relación m/z en el Q_3.

-*Barrido de pérdidas neutras (Neutral Loss Scan),* en el que se realiza un barrido de todos los iones en desfase en ambos cuadrupolos, tras su fragmentación en q_2. Se selecciona un valor de masa de diferencia entre ambos cuadrupolos, de modo que se determinan los analitos que presenten la masa neutra determinada.

En el modo de adquisición de iones hay que diferenciar entre:

-*Adquisición de un ion seleccionado (SIM),* en el que se selecciona un ion específico en el Q_1 siendo el único que llega al detector.

-*Adquisición de una reacción seleccionada (SRM) o de adquisición de una reacción múltiple (MRM),* donde el ion precursor seleccionado en el Q_1, se fragmenta en q_2 originando iones producto, de los cuales solo llega al detector el que se selecciona en el Q_3 *(Loro-Ferrer et al., 2001; Niessen, 2007; Gismera et al., 2009).*

El empleo de cromatografía líquida con espectroscopía de masas en tándem consigue aumentar tanto la sensibilidad como la selectividad de los métodos de análisis, asegurando que el compuesto determinado (incluso a muy bajos niveles de concentración) es el analito buscado. Esto evita, en general, tener que realizar separaciones cromatográficas exhaustivas, incluso en compuestos de polaridades muy similares que coeluyen entre sí. De esta forma, podemos obtener una "doble separación", una producida en la columna cromatográfica y la otra en el analizador, siendo capaces de diferenciar analitos con idéntico tiempo de retención pero con distintas transiciones y viceversa. Muchos autores han escogido LC-MS para la determinación de triazinas en aguas y otras matrices ambientales *(Ferrer et al., 2005; Ruggieri et al., 2005; Postigo et al., 2010; Ricart et al., 2010; Köck-Schulmeyer et al., 2012; Masiá et al., 2013; Homazava et al., 2014; Rocha et al., 2015)* y también se ha empleado UPLC-MS/MS para la determinación de triazinas y alguno de sus productos de degradación *(Gervais et al., 2008; Benvenuto et al., 2010; Hurtado-Sánchez et al.,*

2013; Chen et al., 2015). Destacar que los espectrómetros de triple cuadrupolo más modernos permiten un rápido cambio de polaridad que hace posible incluir pesticidas sensibles a ionización electrospray en modo positivo (ESI+) y en modo negativo (ESI-) en un mismo método de análisis *(Homazava et al., 2014).*

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CAPÍTULO II

AGUAS

INTRODUCCIÓN

En el capítulo II se proponen dos nuevos métodos analíticos para la determinación de triazinas y uno para la determinación simultánea de triazinas y sus productos de degradación en agua de mar, cumpliendo con los criterios establecidos en la legislación vigente en el momento del estudio. Para cada método se recoge la optimización, el estudio de validación y la aplicación a muestras para cada metodología propuesta, comentando sus ventajas y aportaciones respecto a los métodos previamente publicados.

En primer lugar, se desarrolló y validó un método basado en la técnica de extracción en fase sólida con cartuchos Oasis HLB combinada con cromatografía de líquidos con detección de red de diodos (HPLC-DAD) para la determinación simultánea de nueve triazinas (Ametrina, Atrazina, Cianazina, Prometrina, Propazina, Simazina, Simetrina, Terbutilazina y Terbutrina) en aguas superficiales. Además, se evaluó la estabilidad que presentan estos compuestos cuando los cartuchos son almacenados a -18 ºC y 4 ºC, tras la etapa de extracción, y la aplicabilidad del método a muestras de agua de río y agua de mar. Finalmente, el método se aplicó al análisis de cincuenta muestras de agua de mar de dos rías gallegas dedicadas a la pesca y cultivo de marisco, ambas de gran interés tanto económico como ambiental (Arousa y Vigo).

A continuación, se desarrolló y validó un método rápido, simple y acorde con los principios de la Química Verde para la determinación simultánea de siete triazinas (Ametrina, Atrazina, Cianazina, Propazina, Simazina, Simetrina y Terbutilazina) en agua de mar. El método se basa en el empleo de microextracción líquido-liquido dispersiva, que permite el uso de menor volumen de muestra y de disolvente que empleando extracción en fase sólida. Como técnica de determinación se seleccionó la cromatografía de líquidos con detección de red de diodos (HPLC-DAD) y los resultados se confirmaron mediante cromatografía de líquidos-espectrometría de masas (LC-ESI-MS/MS). El método desarrollado se aplicó al análisis de muestras de agua de mar de diez puntos susceptibles de contaminación por triazinas de la ría de A Coruña.

Por último, se recoge el trabajo realizado en una estancia predoctoral de investigación de ámbito internacional, en la que se comenzó el estudio de los principales productos de degradación de las triazinas. La estancia se realizó en Porto (Portugal) en el Instituto da Água da Região Norte (IAREN) bajo la dirección de la Dra. María Fátima Alpendurada con una duración de tres meses (21/9/2015 a 21/12/2015). El trabajo realizado durante la estancia en el IAREN se centró en el desarrollo de un método para la determinación simultánea de las nueve triazinas y sus principales productos de degradación (DEA, DEDIA, DEHA, DET, DIA, DIHA, HA y HT) en muestras de agua de mar empleando extracción en fase sólida on-line y como técnica de

determinación cromatografía de líquidos de ultra resolución-espectrometría de masas en tándem (UPLC-MS/MS). Se llevó a cabo la optimización de los procesos de extracción, separación, identificación y cuantificación de los diecisiete compuestos y la validación del método. Finalmente, el método propuesto fue aplicado al análisis de los compuestos de interés en muestras de agua de mar de diez playas de Matosinhos (Portugal) próximas a zonas de horticultura.

Publicaciones:

Application of a developed method for the extraction of triazines in surface waters and storage prior to analysis to seawaters of Galicia (Northwest Spain). (2013). Rodríguez-González, N., Beceiro-González, E., González-Castro, M.J., Muniategui-Lorenzo, S. *The Scientific World Journal.* http://dx.doi.org/10.1155/2013/536369. **Anexo I-I, página 281.**

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On-line solid-phase extraction method for determination of triazine herbicides and degradation products in seawater by Ultra-Pressure Liquid Chromatography-Tandem Mass Spectrometry. (2016). Rodríguez-González, N., Beceiro-González, E., González-Castro, M.J., Alpendurada. M.F. *Journal of Chromatography A.* 1470, 33-41. **Anexo I-III, página 301.**

Congresos:

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On-line solid-phase extraction method for determination of triazine herbicides and degradation products in seawater by Ultra-Pressure Liquid Chromatography-Tandem Mass Spectrometry. (2016). Rodríguez-González, N., Beceiro-González, E., González-Castro, M.J., Alpendurada. M.F. *"9th European Conference on Pesticides and Related Organic Micropollutants in the Environment" and "15th Symposium on Chemistry and Fate of Modern Pesticides"*. Páginas 178-179. Santiago. (ISBN: 978-989-20-3262-7). **Anexo II-II, página 359.**

APPLICATION OF A DEVELOPED METHOD FOR THE EXTRACTION OF TRIAZINES IN SURFACE WATERS AND STORAGE PRIOR TO ANALYSIS TO SEAWATERS OF GALICIA (NW SPAIN)

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ABSTRACT

A simple method based on solid-phase extraction combined with liquid chromatography for simultaneous determination of nine triazine herbicides (ametryn, atrazine, cyanazine, prometryn, propazine, simazine, simetryn, terbuthylazine and terbutryn) in surface water samples was developed and validated. Under optimized conditions, 50 mL of water sample was pumped through the Oasis HLB cartridge and triazines were eluted with 3 mL acetone. Finally the extract was concentrated to dryness, re-constituted with 1 mL methanol:water (1:1) and injected into the HPLC-DAD system. The stability of the herbicides on the cartridges at -18 and 4ºC was also evaluated and the recoveries obtained after three weeks of storage were satisfactory for all compounds. The analytical features of the proposed method were satisfactory: repeatability and intermediate precision were < 10% and recoveries in spiked river water and seawater samples was higher than 93 % for all compounds studied. Limits of quantification (varied from 0.46 to 0.98 µg L^{-1}) were adequate allowing the determination of these compounds at the levels requested by the 2008/105/EC Directive. Finally, this method was applied to the analysis of 50 seawater samples from Galicia (NW Spain).

1. Introduction

Triazines are recognised herbicides which have been broadly used in agriculture over the recent decades. The surface water receives fluxes of these compounds mainly of agricultural origin, due to their widespread use in this field [1]. Their high persistence and toxicity have required rigorous control of environmental contamination. Therefore, the presence of pesticides in surface waters is regulated by the European Directive 2008/105/EC that establishes a maximum permitted concentration of 2 and 4 µg L^{-1} for atrazine and simazine respectively [2]. It is important to take into account that atrazine and simazine have been included in the list of "priority hazardous substances" in Decision 2455/2001/EC [3] that amends the Directive 2000/60/EC [4] and atrazine, ametryn, prometryn, terbutryn, simazine and propazine are considered as a group to be

endocrine-disrupting chemicals by the U.S. Environmental Protection Agency [5]. Thus, sensitive methods for determining the low concentrations of triazine herbicides in environmental samples are required.

Different chromatographic techniques have been used to determine triazines. Gas chromatography coupled to mass spectrometry has been widely employed [6-11] and the use of liquid chromatography with different detectors such as ultraviolet [12-16], diode array [17, 18] or mass spectrometry [19-25] have also been reported. An extraction procedure to preconcentrate the analytes and remove possible interferences is mandatory to achieve the required levels. For this purpose, solid phase extraction (SPE) is the preconcentration technique most commonly used for the determination of triazines in water samples [7-11, 17, 19-23, 25]. Recently, micro-extraction techniques have become important procedures in environmental analysis. Thus, some micro-extraction methods such as solid phase micro-extraction (SPME) [26], stir bar sorptive extraction (SBSE) [6], liquid phase micro-extraction (LPME) [24], liquid-liquid-solid micro-extraction (LLSME) [13] and dispersive liquid-liquid micro-extraction (DLLME) [14, 18, 27] have been applied for extraction and preconcentration of triazines in water as alternative to the SPE techniques. However, some of these new techniques have drawbacks like low sensitivity for the triazines studied [26], poor recoveries [14, 18] and in many cases they are very laborious [13, 24].

The aim of the current study was to develop a simple, sensitive and low-cost method based on SPE for the extraction of nine triazines from surface water samples and the storage of the compounds until their determination by HPLC-DAD. Samples of river water and seawater were chosen to illustrate the reliability of the method. Finally, the method was applied to analyze 50 samples of seawater from two areas dedicated to shellfishing and fishing.

2. Experimental

2.1. Site location and sampling

Fifty seawater samples from Galicia (NW Spain) were collected at two different locations (zone 1 and 2) from April to June 2011. The health of both estuaries is a priority for the government of the region, because they are engaged in shellfish and fishing. In zone 1 (estuary of Arousa Island) 37 sites were selected for sampling and in zone 2 (estuary of Vigo) 13 sites were selected. The sampling locations and their designations are shown in Figure 1. At each sampling locations three samples were collected.

Samples were collected in amber glass bottles and transported to the laboratory under cooled conditions (4ºC). Upon reception, samples were filtered through 0.6 µm glass

fibre filters to eliminate suspended solid matter and the solid-phase extraction was carried out. The cartridges were stored at -18ºC in the dark until analysis.

2.2. Chemical and materials

All herbicides analytical standards were supplied by Sigma-Aldrich (Inc. St. Louis, MO, USA). The individual stock standard solutions of 1000 mg L^{-1} were prepared in methanol by exact weighing of high-purity substances and stored at –18ºC in the dark. Then a mixture of all the compounds was prepared in methanol containing 10 mg L^{-1} each individual triazine and stored at -18ºC. All working solutions were daily prepared by appropriate dilution of the 10 mg L^{-1} standard solutions with methanol:water (1:1, v/v).

Acetonitrile was purchased from Panreac (Barcelona, Spain) and methanol and acetone from Romil (Cambridge, UK). All chemicals were HPLC grade. Ultra-pure Milli-Q water was obtained using a Millipore Milli-Q system (Millipore, Bedford, MA, USA).

Reverse phase polymeric cartridges Oasis HLB (6 mL, 200 mg) were supplied by Waters (Milford, MA, USA). Glass fibre MN GF-6 filters (0.6 µm pore size) were purchased from Macherey Nagel (Düren, Germany) and 0.20 µm pore-size nylon membrane filters from Millipore (Bedford, MA, USA). A visiprep vacuum system from Supelco (Bellefonte, PA, USA), a rotary evaporator (Büchi, Labortechnic AG, Flawil, Switzerland) and an ultrasonic bath (Branson 3200, Energieweg, The Netherlands) were used.

Uncontaminated river and seawater samples collected from a brook and Riazor beach in the city of A Coruña (Galicia, NW Spain) were used for the validation of the method.

2.3. Instrumental analysis

Chromatographic analyses were performed using a high performance liquid chromatography-diode array detector (HPLC-DAD). The system consisted of a 2695 pump with a 996 Diode Array Detector from Waters (Milford, MA, USA). The column was a stainless steel column (150 mm x 4.6mm ID, particle size 5 µm) packed with Hypersil GOLD C_{18} chemical bonded phase from Thermo Scientific (Austin, TX, USA).

The analysis was carried out using the following gradient elution: acetonitrile initial percentage of 30% (8 min) increased linearly to 40% in 5 min; increased to 50% in 5 min, after which the percentage was returned to the initial conditions in 9 min. The flow rate was 1 mL min^{-1}, the oven temperature was set at 25ºC and 20 µL of sample volume was used.

The absorbance was measured continuously in the 200-400 nm range and peaks areas quantification were carried out at 222.7 nm in order to achieve maximum sensitivity.

All triazine herbicides were identified initially by retention time and then by applying spectral contrast techniques (incorporated in Millenium[32] software) homogeneity of the spectral peak was confirmed. Finally a spectral identification was carried out contrasting the spectrum with a standard library created in wavelength interval of 200-400 nm.

2.4. Solid phase extraction procedure

The extraction was performed as follows: the cartridge was conditioned by washing it with 10 mL methanol and 10 mL Milli-Q water. Water sample (50 mL) was pumped through the cartridge at a flow rate of 10 mL min^{-1} and then the cartridge was washed with 20 mL Milli-Q water. Once the retention step had been completed, the cartridge was partially dried under a vacuum system for 5 min and then it was totally dried using a nitrogen stream for 30 min. The elution of retained compounds was done with 3 mL of acetone and the organic extract was brought to complete dryness under a combination of rotary evaporator at 40ºC and a gentle nitrogen stream. Finally the sample was reconstituted in methanol/water (1:1, v/v) to a final volume of 1 mL and injected into the HPLC.

3. Results and discussion

3.1. SPE method optimization

The solid phase extraction procedure was based on a previous method for drinking water developed by the same authors [28]. To optimize the method for surface waters, a filtration step prior to the SPE was studied. For this purpose two different filters were assayed (glass microfiber filters and nylon membrane filters). A volume of 50 mL of seawater sample was spiked at a concentration level of 2 µg L^{-1} (lowest legislation level).

The spiked sample was mixed in an ultrasonic bath for 5 min to ensure efficient distribution of the herbicides and it was allowed to equilibrate for 5 min prior to extraction and then it was filtered. Isolation and determination of the compounds from the spiked samples was performed as described above.

Results showed an important decrease on the recovery for simetryn (68%) and terbutryn (50%) when nylon membrane filter was employed (recoveries between 82 and 101% were obtained for the other seven herbicides). However, values of recovery were satisfactory for all compounds when glass microfiber filter was used.

3.2. Stability of herbicides on the cartridges

Due to herbicides may be degraded during storage of the sample at 4ºC by processes such as hydrolysis or microbial decomposition, the fact of being able to perform the storage of the cartridges after the solid-phase extraction for extend time periods is

extremely useful. Thus, the extraction can be performed on the day of sampling or even at the time of sampling for further transport to the laboratory. For this reason, the stability of the herbicides loaded on Oasis HLB cartridges was investigated under different storage conditions. Several cartridges were loaded with 50 mL of seawater spiked at 2 µg L^{-1} with a standard mixture of the nine herbicides and stored at 4 and -18ºC. Elution of triazines was carried out after 1, 2, 3, 6 and 8 weeks of storage employing three cartridges for each time and temperature studied. Before elution cartridges kept at –18ºC and 4ºC were defrosted at room temperature for 2 and 1 h respectively. The organic extracts obtained were reconstituted and analyzed.

The effect on the recovery of the herbicides was evaluated. The results are shown in Figure 2. As it can be seen (Figure 2a), triazines showed a high stability at -18ºC being the recoveries quantitatives after 6 weeks of storage for all the compounds (recovery values higher than 90% and RSD < 10%), except atrazine and terbutryn in which case recovery considerably decreased after 3 weeks (80 and 76% respectively after 6 weeks of storage). However, the recovery of the compounds was only quantitative up to 3 weeks at 4ºC, and it was decreasing gradually for higher storage periods of time, mainly for prometryn, propazine and terbutryn (Figure 2b).

On the other hand, a major variability on the recoveries was observed at 4ºC. Therefore, it can be concluded that the most reliable method for storing the herbicides on the cartridge is keep them at –18 ºC during 3 weeks because the integrity of the analytes is not affected.

3.3. Method validation

The analytical characteristics of the SPE-HPLC method were evaluated using a 50 mL of uncontaminated seawater sample spiked with a standard mixture of the compounds. The linearity was studied at 6 concentration levels (0.5, 1, 1.5, 2, 2.5 and 3 µg L^{-1}). As it can be seen in Table 1, determination coefficients (r^2) were higher than 0.991 for all the herbicides at concentrations within the interval tested.

The limits of detection (LODs) were determined as $3*S_{y/x}/b$ and the limits of quantification (LOQs) as $10*S_{y/x}/b$, where $S_{y/x}$ is the residual standard deviation and b is the slope of the calibration curves. As it can be seen in Table 1, the detection and quantification limits between 0.15-0.33 and 0.46-0.98 µg L^{-1} respectively were adequate, being the LOQs much lower than parametric value requested by the legislation for surface water [2].

Repeatability and intermediate precision were evaluated at 2 µg L^{-1}. The repeatability was calculated as within-day RSD of peak areas using eight replicates analyzed in the same day and by the same analyst. In the case of intermediate precision five replicates were analyzed in consecutive days and by the same analyst and it was calculated as

between-day RSD of peak areas. As it can be seen in Table 1, the results obtained were satisfactory with RSD values below 10% for all compounds in both cases.

The accuracy (expressed as percent recovery) of the method was studied using seawater sample spiked with 2 μg L^{-1} of a standard mixture of the compounds. The recoveries obtained for five replicates (n=5) of the sample spiked with the triazine herbicides are presented in Table 2. The results demonstrated that the method achieved satisfactory recoveries in the range of 93-106%, with associate standard deviations below 9% for all compounds.

Furthermore, a river water sample was also used to evaluate the reliability of the SPE-HPLC method. For this purpose, five replicates of 50 mL of sample spiked at 2 μg L^{-1} with a standard mixture of the compounds were subjected to the optimized method and analytical recoveries were evaluated. The results obtained (see Table 2) have shown to be satisfactory with recoveries in the range of 95-104%, with RSD below 7% for all compounds.

Therefore, it can be concluded that the proposed method is useful to determine triazines in surface water samples. As an example, chromatograms corresponding to seawater (Figure 3a) and seawater sample spiked at a concentration level of 2 μg L^{-1} (Figure 3b) are presented in Figure 3.

3.4. Application

Finally, the method was applied to the analysis of 50 seawater samples from Galicia (NW Spain). Although the herbicides under study have not been detected in the samples analyzed, studies to generate information related to their levels in areas where seafood is growing (mussels, crabs, oysters…) are of great economic and environmental interest.

4. Conclusions

The proposed method provides a simple and inexpensive way for simultaneous determination of nine triazine herbicides in surface waters. Furthermore, it uses small volume of organic solvents in agreement with the principles of Green Chemistry. The stability of the herbicides on the cartridge stored at -18ºC is of great interest and usefulness since the use of the cartridges allows the storage of the triazines until analysis, avoiding the problem associated with the maintaining herbicides integrity in aqueous solution when long periods of storage are required before analysis. This fact makes the solid-phase extraction procedure developed as a promising alternative to conventional water sampling for triazines analysis. However, further work should be necessary to optimize the extraction methodology “in-situ” during sampling.

The method was successfully applied for river and seawater samples and satisfactory precision, accuracy and sensitivity were obtained. Using 50 mL of seawater sample, the LOQs obtained were lower than the parametric value requested by the legislation [2].

The method was applied to the analysis of 50 seawater samples from Galicia (NW Spain).

Although the triazines under study have not been detected in the samples analyzed, the monitoring of their levels in marine ecosystems is of great economic and environmental importance. It is important to take into account that measurements of known quality represent the foundation of the water quality evaluation system and the basis for decisions to be taken to achieve Marine Strategy Framework Directive and environmental objectives at the end of 2015 [29].

Finally, it is noteworthy that methods based on solid-phase extraction combined with liquid chromatography have been commonly used to measure triazines in drinking and ground waters; however, there are not studies in seawater.

Acknowledgments

The authors acknowledge the financial support of the "Ministerio de Educación y Ciencia" (Project CTQ2007-63949/BQU) and the "Xunta de Galicia" (Project 09MDS038103PR). N. Rodríguez-González also thanks to the "Xunta de Galicia" for the concession of her award under the program "Campus del mar".

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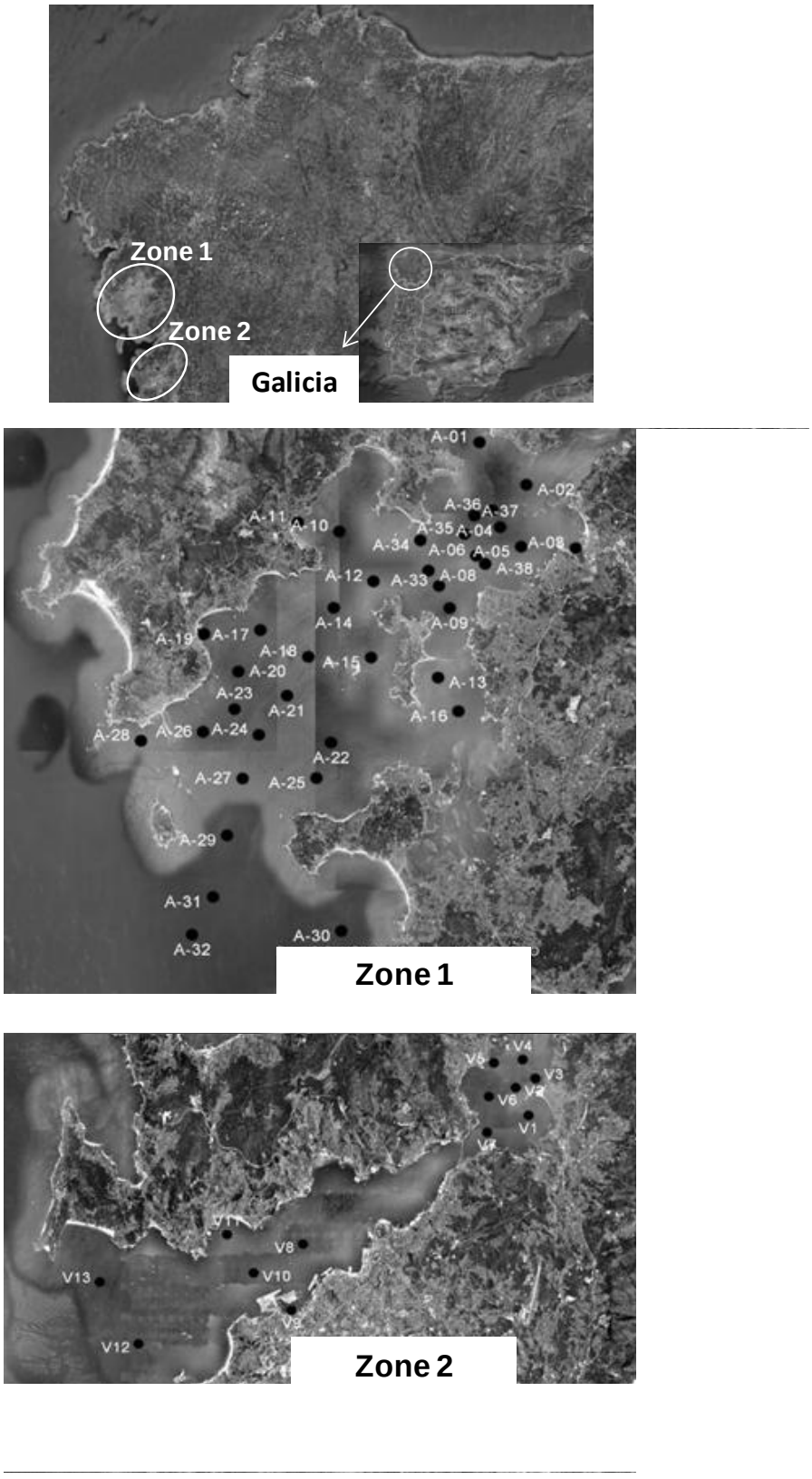
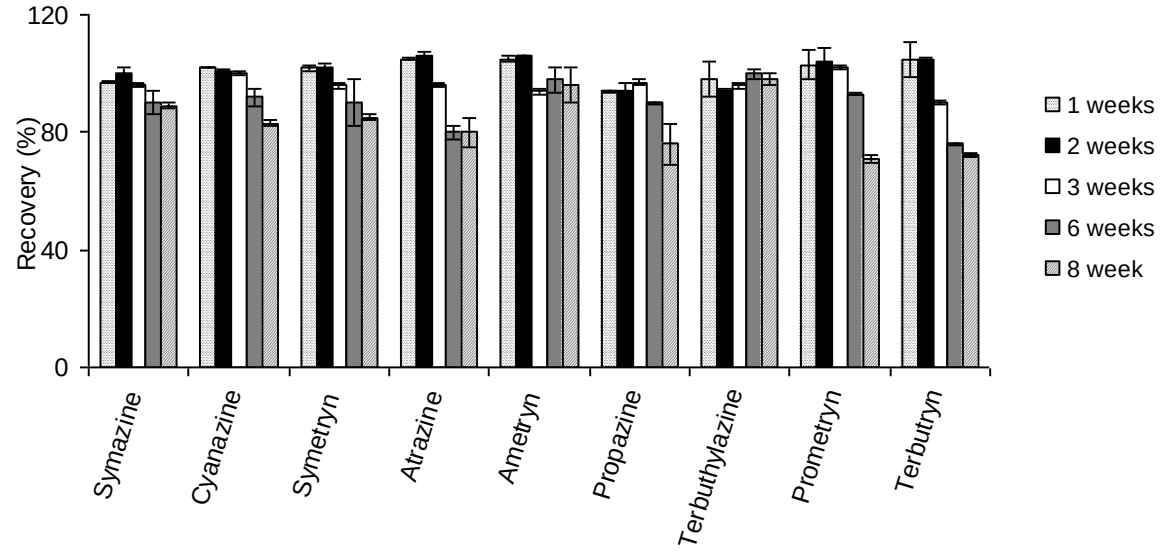


Figure 1. Map of sampling sites location at Galicia (NW Spain)

(a)



(b)

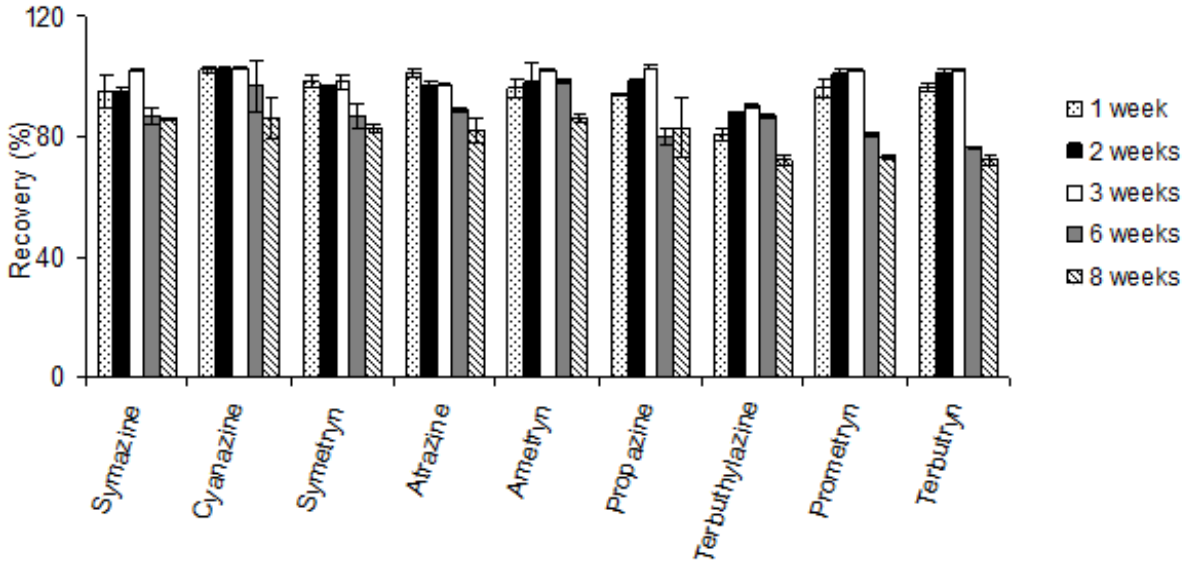


Figure 2. Effect of storage conditions on the recovery of the herbicides. (a) temperature -18ºC, (b) temperature 4ºC

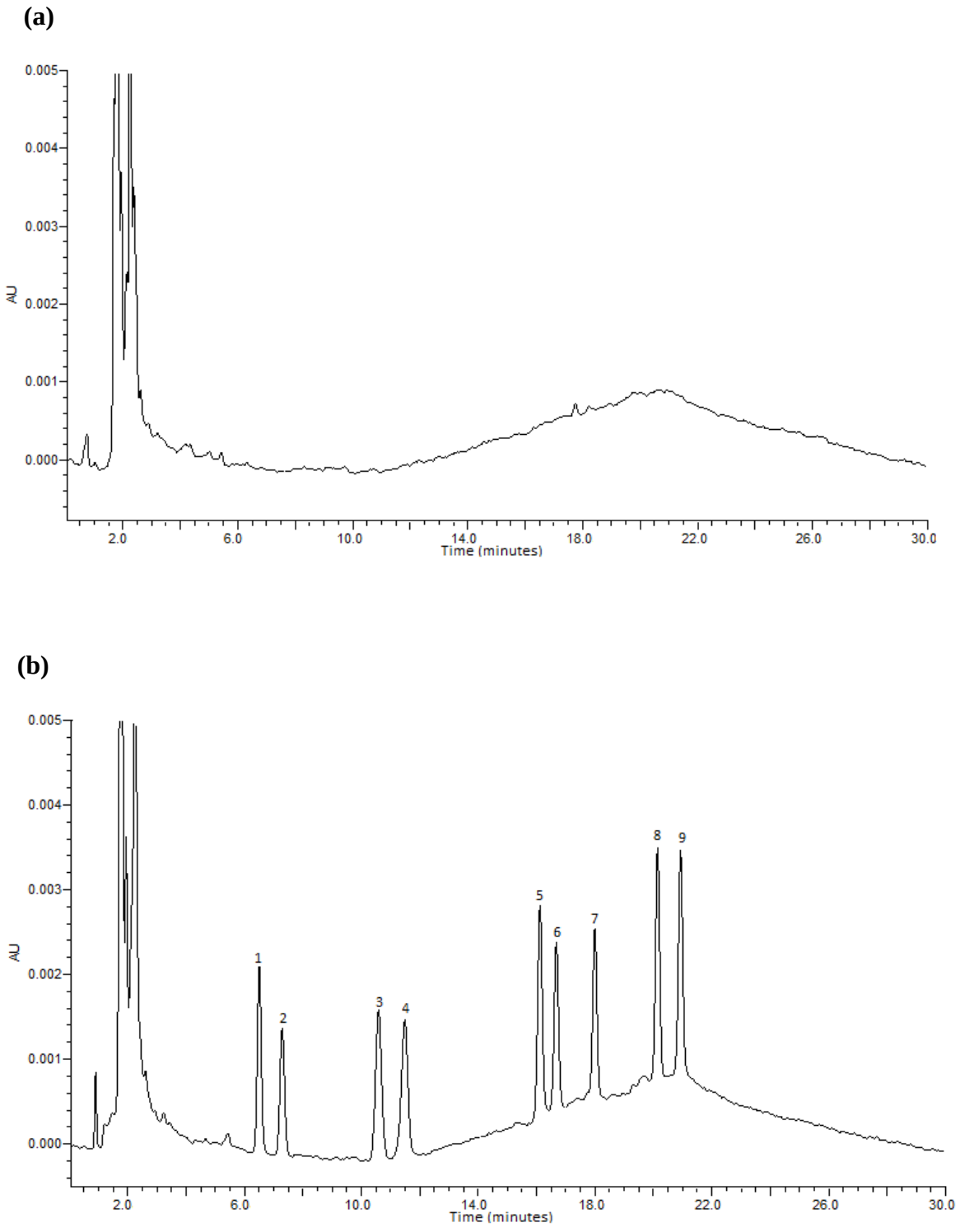


Figure 3. Chromatograms obtained after solid phase extraction. (a) seawater sample, (b) seawater sample spiked at 2 µg L^{-1}. Target compounds are numbered as follows: (1) simazine, (2) cyanazine, (3) simetryn, (4) atrazine, (5) ametryn, (6) propazine, (7) terbuthylazine, (8) prometryn, (9) terbutryn

Table 1. Analytical characteristics of the SPE-HPLC method

Compound	LOD (µg L^{-1})	LOQ (µg L^{-1})	Determination coefficient (r^2)	Repeatability [a] RSD (%)	Reproducibility [a] RSD (%)
Simazine	0.28	0.84	0.9962	4.3	10.0
Cyanazine	0.33	0.98	0.9910	4.4	4.8
Simetryn	0.23	0.70	0.9955	5.7	6.5
Atrazine	0.15	0.46	0.9981	5.5	7.6
Ametryn	0.28	0.85	0.9915	5.2	5.2
Propazine	0.18	0.56	0.9978	4.5	8.6
Terbuthylazine	0.26	0.80	0.9948	7.6	8.3
Prometryn	0.16	0.50	0.9982	1.4	2.4
Terbutryn	0.26	0.80	0.9966	3.1	5.1

[a] n = 8 and n = 5 for repeatability and reproducibility respectively (2 µg L^{-1})

Table 2. Study of analytical recovery in river and sea waters (2 µg L^{-1}, n=5)

	Analytical recovery (%)	± RSD (%)
Compound	River water	Seawater
Simazine	100 ± 7.0	100 ± 7.8
Cyanazine	100 ± 6.1	106 ± 8.8
Simetryn	100 ± 3.1	103 ± 9.0
Atrazine	96 ± 5.3	93 ± 8.9
Ametryn	100 ± 5.1	102 ± 7.3
Propazine	99 ± 5.4	103 ± 8.7
Terbuthylazine	104 ± 2.5	99 ± 8.4
Prometryn	96 ± 6.6	106 ± 6.4
Terbutryn	95 ± 4.1	99 ± 2.3

SIMULTANEOUS DETERMINATION OF NINE TRIAZINES IN SEAWATER BY SOLID-PHASE EXTRACTION FOLLOWED BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-DIODE ARRAY DETECTOR

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INTRODUCTION

The high persistence of pesticides has required rigorous control of environmental contamination. Therefore, the presence of pesticides in surface waters is regulated by the European Directive 2008/105/EC [1] that establishes as maximum permitted concentration 2 and 4 $\mu g L^{-1}$ for atrazine and simazine respectively. It is important to take into account that atrazine and simazine have been included in a list of "priority hazardous substances" (Decision n.o 2455/2001/EC [2], that amends the Directive 2000/60/EC [3]) due to their persistence, toxicity, moderate leaching capacity and potential to adsorb onto soils and sediments.

The aim of this work was the comparison of two mobile phases for the simultaneous determination of nine triazines in water by SPE-HPLC with DAD detection, as well as the application of the most suitable method to the analysis of seawater samples.

EXPERIMENTAL

HPLC-DAD Determination

HPLC-DAD Waters with a column packed with Hypersil GOLD C_{18} (150 mm x 4.6 mm ID, 5 µm)

Absorbance measured continuously at 200-400 nm range. Quantification at 222.7 nm

Gradient elution ACN-H_2O

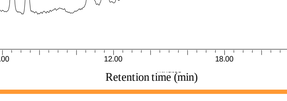
Initially 30% ACN (8 min) increased to 40% in 5 min, to 50% in 5 min and returned to initial conditions in 9 min

Isocratic elution MeOH

MeOH-0.002 M CH_3COONH_4 buffer pH 3.2

SPE Procedure

Cartridge Oasis HLB
Tube 6 ml (200 mg)
+
3 ml acetone
elution solvent



Dryness

Rotary evaporator
+
Gentle N_2 stream

Dilution

1 ml methanol:water (1:1)

Sample site location

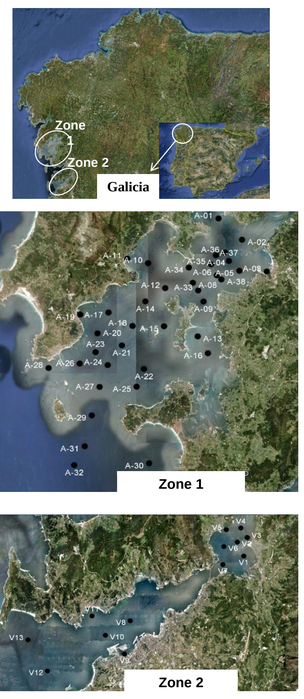
Samples were collected at two locations:

Zone 1: estuary of Arousa Island

Zone 2 (estuary of Vigo)

Upon reception samples were filtered (glass fiber filters) and SPE carried out

Cartridges were stored at - 20 ºC



RESULTS AND DISCUSSION

Optimization of chromatographic conditions

As it can be seen in **Figure 1** the use of gradient elution of ACN-H_2O at the optimized conditions allowed an adequate separation with a good resolution of the nine compounds

Figure 1 Chromatogram using gradient elution of ACN-H_2O (50 µg L^{-1})
(1) simazine, (2) cyanazine, (3) simetryn, (4) atrazine, (5) ametryn, (6) propazine, (7) terbuthylazine, (8) prometryn, (9) terbutryn

The effect of different pH was studied for isocratic elution using 55% MeOH-45% 0.002 M ammonium acetate buffer. A satisfactory separation was only obtained using a pH = 3.2 (see **Figure 2**)

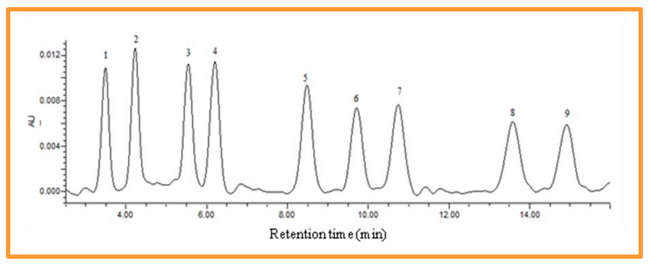


Figure 2 Chromatogram using isocratic elution of MeOH-0.002 M acetate buffer at pH 3.2 (1 mg L^{-1})
(1) cyanazine, (2) simazine, (3) simetryn, (4) atrazine, (5) ametryn, (6) propazine, (7) terbuthylazine, (8) prometryn, (9) terbutryn

Analytical characteristic were studied. LOD and LOQ are shown in **Table I** Reproducibility and repeatability (n=10) were satisfactory with values lower than 4.5 and 3.5% respectively

lower LOQ

Table I: LOD and LOQ

Gradient ACN-H_2O method			
Compound	t_r (min)	LOD (µg L^{-1})	LOQ (µg L^{-1})
Simazine	6.58	4	12
Cyanazine	7.40	15	45
Simetryn	10.72	15	47
Atrazine	11.68	7	17
Ametryn	16.24	10	31
Propazine	16.83	15	46
Terbuthylazine	18.15	11	35
Prometryn	20.26	4	12
Terbutryn	21.05	5	16
MeOH/0.002 M ammonium acetate method			
Compound	t_r (min)	LOD (µg L^{-1})	LOQ (µg L^{-1})
Cyanazine	3.48	74	225
Simazine	4.22	81	247
Simetryn	5.53	98	297
Atrazine	6.20	72	217
Ametryn	8.47	157	475
Propazine	9.71	146	442
Terbuthylazine	10.72	87	265
Prometryn	13.56	83	252
Terbutryn	14.91	115	350

Study of SPE procedure

Table II: Analytical characteristics (V =500 mL)

SPE-gradient ACN-H_2O method				
Compound	LOD (µg L^{-1})	LOQ (µg L^{-1})	Repeatability RSD (%)	Reproducibility RSD (%)
Simazine	0.012	0.035	4.3	11.0
Cyanazine	0.010	0.032	4.4	4.8
Simetryn	0.017	0.052	5.7	6.5
Atrazine	0.011	0.035	5.5	7.6
Ametryn	0.013	0.039	5.2	5.2
Propazine	0.023	0.069	4.5	8.6
Terbuthylazine	0.022	0.068	7.6	8.3
Prometryn	0.012	0.037	1.4	2.4
Terbutryn	0.013	0.040	3.1	5.1
SPE- MeOH/0.002 M ammonium acetate method				
Compound	LOD (µg L^{-1})	LOQ (µg L^{-1})	Repeatability RSD (%)	Reproducibility RSD (%)
Cyanazine	0.20	0.60	2.5	2.6
Simazine	0.28	0.85	4.6	6.6
Simetryn	0.37	1.11	2.6	8.3
Atrazine	0.17	0.52	6.2	6.9
Ametryn	0.46	1.39	2.0	5.2
Propazine	0.20	0.61	5.1	6.7
Terbuthylazine	0.59	1.79	6.0	8.6
Prometryn	0.26	0.79	4.1	7.0
Terbutryn	0.20	0.60	6.2	12

A procedure based on SPE employing Oasis HLB and 500 mL of water was used prior to the chromatographic determination. The overall procedure was satisfactory in both cases with an adequate repeatability and reproducibility (2 µg L^{-1}, legislation level) (see **Table II**)

LOQs allow the determination of triazines in surface waters at levels required by the legislation

The volume of sample used can be considerably reduced when ACN-H_2O method is employed

ACN-H_2O method was chosen for real samples application

Application to seawater samples

The SPE-HPLC method using ACN-H_2O was applied to the determination of triazines in seawater using 50 mL of sample. A study of sensitivity was carried out and repeatability and recoveries were calculated at 2 µg L^{-1} levels (n=5). Results are presented in **Table III**

LODs, LOQs and recoveries were adequate being LOQs much lower than the parametric value requested by the legislation

Table III: Analytical characteristics

Seawater (V = 50 mL)				
Compound	LOD (µg L^{-1})	LOQ (µg L^{-1})	Recovery (%)	
Simazine	0.28	0.84	100	9.8
Cyanazine	0.33	0.99	106	8.8
Simetryn	0.23	0.70	103	9.0
Atrazine	0.15	0.46	93	9.3
Ametryn	0.28	0.85	102	7.3
Propazine	0.18	0.56	103	8.7
Terbuthylazine	0.26	0.80	99	8.4
Prometryn	0.16	0.50	106	6.4
Terbutryn	0.26	0.80	99	2.3

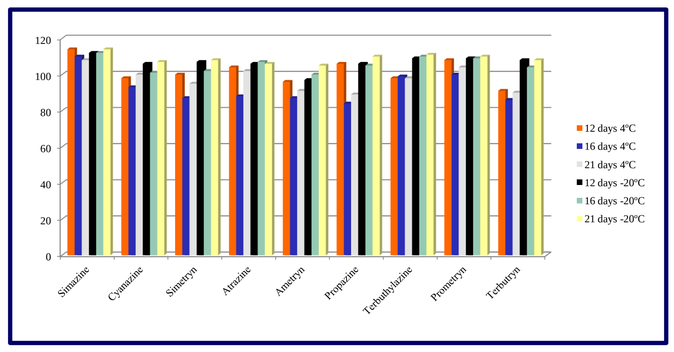


Figure 3 Stability of herbicides stored on Oasis HLB cartridges 50 mL of seawater spiked to 2 µg L^{-1}

The effect of different storage temperature (4 and – 20 ºC) and time (12 - 21 days) on the recovery after storing herbicides in the cartridge was evaluated. Recoveries are presented in **Figure 3**.

Storing the cartridges at – 20 ºC were satisfactory independently of the storage time

Storing at 4 ºC was adequate during 12 days however, RSD were very high (12-22%) and recoveries < 70% for some compounds in any cartridges after 16 and 21 days

The method was applied to seawater samples. Although, none of the herbicides were detected in the 51 seawater samples from Vigo and Isla de Arosa estuaries, it is important monitoring its levels in these areas because they are engaged in shellfish and fishing area.

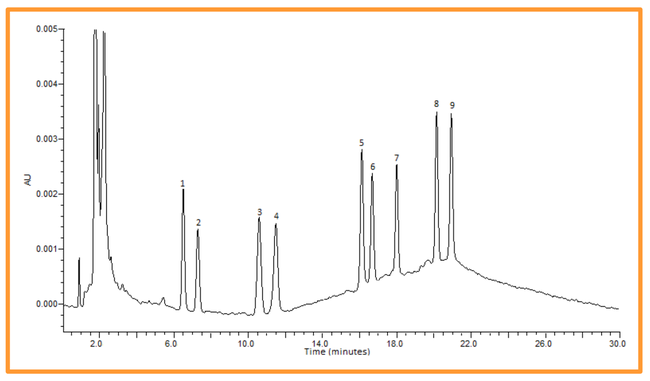


Figure 5 Chromatogram of seawater sample spiked at 2 µg L^{-1}
(1) simazine, (2) cyanazine, (3) simetryn, (4) atrazine, (5) ametryn, (6) propazine, (7) terbuthylazine, (8) prometryn, (9) terbutryn

In **Figure 4 and 5** chromatograms of a seawater and spiked seawater samples respectively are shown

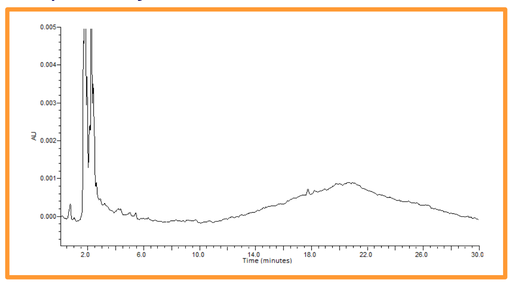


Figure 4 Chromatogram of a seawater sample obtained after solid phase extraction

CONCLUSIONS

An effective, accurate, simple and low-cost method based on SPE with Oasis HLB combined with HPLC-DAD for the simultaneous analysis of triazines in seawater was developed. Although, the use of ACN-water and MeOH-0.002 M ammonium acetate buffer as mobile phase allowed the quantification of the target compounds at the levels required by the surface water legislation, a sample volume of 50 mL can be employed when ACN-water was used. The proposed method for determining triazine herbicides in waters could be established as a suitable method to routine analysis making solid-phase extraction upon reception the samples and storing the cartridges at -20 ºC in the dark until analysis.

REFERENCES

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[3] Council of the European Communities, 2000. Directive 2000/60/EC establishing a framework for community action in the field of water policy. Off. J. Eur.Union. L 327, 1-73.

AN ENVIRONMENTAL FRIENDLY METHOD FOR THE DETERMINATION OF TRIAZINE HERBICIDES IN ESTUARINE SEAWATER SAMPLES BY DISPERSIVE LIQUID-LIQUID MICROEXTRACTION

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ABSTRACT

A fast, simple, sensitive and green chemistry method using dispersive liquid-liquid microextraction (DLLME) for the simultaneous determination of seven triazine herbicides (ametryn, atrazine, cyanazine, propazine, simazine, simetryn and terbuthylazine) in estuarine seawater samples has been developed. DLLME was carried out using a small volume of seawater (25 mL) and 300 µL of 1-octanol. Herbicide concentrations were determined by liquid chromatography-diode array detection and results were confirmed by liquid chromatography-electrospray ionization tandem spectrometry analysis (LC-ESI-MS/MS). The analytical features of the proposed method were satisfactory with repeatability < ±5%, intermediate precision < ±10% and recoveries ranged from 81-102% for all compounds. All the triazines exhibited linear matrix calibration curves with coefficients of determination > 0.999 for all the analytes except for simazine (0.9975). Limits of quantification ranged between 0.19 and 1.12 µg L^{-1}. The method was applied to the analysis of seawater samples from ten points susceptible to contamination by triazines from estuary of A Coruña (Galicia, NW of Spain). The levels of the seven triazines were below the LODs in the analyzed samples. Use of proposed method will allow for monitoring of triazines at levels below the regulatory limits set by the European Directive 2008/105/EC of 2 and 4 µg L^{-1} for atrazine and simazine, respectively.

Keywords: Triazine herbicides; Seawater samples; Dispersive liquid-liquid microextraction; HPLC-DAD; LC-ESI-MS/MS.

1. Introduction

Triazines are well-known herbicides that are worldwide applied to soil for the control of weeds in many agricultural crops, as well as, railways, roadside and golf courses. The marine environment receives fluxes of these compounds mainly of agricultural origin. These compounds are highly resistant and can survive many years in soil, water and organisms. Therefore, they are considered an important class of chemical pollutants.

Atrazine and simazine are included in the group of endocrine-disrupting compounds by the US Environmental Protection Agency (2009). Furthermore, the presence of pesticides in surface waters is regulated by the European Directive 2008/105/EC that establishes a maximum permitted concentration of 2 and 4 µg L^{-1} for atrazine and simazine respectively (Council of the European Communities 2008a). It is important to take into account that atrazine and simazine have been included in the list of "priority hazardous substances" in Decision 2455/2001/EC (Council of the European Communities 2001) that amends the Water Framework Directive 2000/60/EC (Council of the European Communities 2000). In order to support the implementation of the Directive 2008/105/EC, fast, simple and sensitive analytical methods are required.

Different chromatographic techniques have been used to determine triazines. Gas chromatography coupled to mass spectrometry has been widely employed (Sánchez-Ortega et al. 2009; Bagheri et al. 2010; Djozan et al. 2010; Katsumata et al. 2010; Matamoros et al. 2010; Navarro et al. 2010; Portolés et al. 2011; Bagheri et al. 2012) and the use of liquid chromatography with different detectors such as ultraviolet (Hu et al. 2009a, Hu et al. 2009b; Zhou et al. 2009; Beale et al. 2010; See et al. 2010), diode array (Kueseng et al. 2009; Wang et al. 2010a; Wang et al. 2011; Rodríguez-González et al. 2013) or mass spectrometry (Mazzella et al. 2009; van Pinxteren et al. 2009; Dujakovic et al. 2010; García-Galán et al. 2010; Postigo et al. 2010; Trtic-Petrovic et al. 2010; Lissalde et al. 2011) was also reported.

An extraction procedure to preconcentrate the analytes and remove possible interferences is mandatory to achieve the required levels. For this purpose, solid phase extraction (SPE) is the preconcentration technique most commonly used for the determination of triazines in water samples (Kueseng et al. 2009; Mazzella et al. 2009; van Pinxteren et al. 2009; Bagheri et al. 2010; Dujakovic et al. 2010; García-Galán et al. 2010; Katsumata et al. 2010; Matamoros et al. 2010; Navarro et al. 2010; Postigo et al. 2010; Lissalde et al. 2011; Portolés et al. 2011; Rodríguez-González et al. 2013). However, this technique is being replaced by other fast techniques that minimize the waste of organic solvents according with the principles of Green Chemistry (Anastas et al. 2010). Thus, some microextraction methods such as solid phase microextraction (SPME) (Mohammadi et al. 2009; Djozan et al. 2010), stir bar sorptive extraction (SBSE) (Sánchez-Ortega et al. 2009), liquid phase microextraction (LPME) (Trtic-Petrovic et al. 2010), liquid-liquid-solid microextraction (LLSME) (Hu et al. 2009b) and dispersive liquid-liquid microextraction (DLLME) (Zhou et al. 2009; Wang et al. 2010; Wang et al. 2011; Sanagi et al. 2012) have been applied for extraction and preconcentration of triazines in water as alternative to the SPE techniques. However, some of these new techniques have drawbacks like low sensitivity for the triazines

studied (Mohammadi et al. 2009), poor recoveries (Zhou et al. 2009; Wang et al. 2010) and in many cases they are very laborious (Hu et al. 2009b; Trtic-Petrovic et al. 2010).

DLLME is based on the extraction of analytes in aqueous samples by employing an appropriated mixture of extraction solvent and disperser solvent to obtain a cloudy solution which assures a high contact surface and analyte extraction. The main problem is the adequate selection of the mixture of solvents because analytes losses can occur. This situation can be avoided by using an agitation step instead of adding the disperser solvent to achieve the formation of the cloudy solution (Zhang et al. 2011). The advantages of DLLME are simplicity of operation, short extraction time, low cost and high enrichment factors (Rezaee et al. 2006).

The aim of this work was the development of an environmental friendly, simple, fast and sensitive method for the simultaneous determination of seven triazine herbicides in estuarine seawater based on dispersive liquid-liquid microextraction (DLLME). The herbicides were determined by liquid chromatography with diode array detection (HPLC-DAD) and confirmed by liquid chromatography-electrospray ionization tandem spectrometry analysis (LC-ESI-MS/MS). This method enables the determination of these pollutants at the levels required by European Union legislation (Council of the European Communities 2008a) using only 25 mL of sample; consequently it can be an important tool to control the presence of triazines in seawater samples. The developed method was employed to determine the concentration of target compounds in seawater samples from estuary of A Coruña (Galicia, NW of Spain).

2. Materials and methods

2.1. Study area and sampling

The area studied is located in the coastline of Galicia (NW of Spain). Seawater samples were collected from 10 potential polluted sites by triazines in estuary of A Coruña (see Fig. 1) during March of 2013. The estuary is formed by the mouth of Mero River and it is also fed by many small rivers and brooks that traverse areas dedicated to agriculture. The sampling locations were selected by their proximity to one or more potential sources of contamination by triazine herbicides.

The study area is illustrated in Fig. 1 and the 10 sampling points and the source in brackets are listed below: 1 (tram line), 2 (golf course), 3 (a railway line and vegetable gardens), 4 (vegetable gardens), 5 (mouth of a river flowing through a golf course and growing areas), 6 (mouth of the Mero river that traverses areas dedicated to agriculture), 7 and 8 (residential areas with gardens and parks), 9 (mouth of two rivers flowing through a campsite, vegetable gardens and residential areas with gardens) and 10 (lake with access to the sea which receives two rivers that cross growing areas, vegetable

gardens, and residential areas with gardens). At each sampling point three samples were collected.

Seawater samples were collected in amber glass containers, filtered through 0.6 µm glass fibre MN GF-6 filters from Macherey Nagel (Düren, Germany) to eliminate suspended solid matter. Due to the low stability of triazines, the samples were analyzed the day of sampling.

Furthermore, unpolluted seawater samples from the Riazor beach at the city of A Coruña (Galicia, NW Spain) were used for the optimization and validation of the method

2.2. Standard and reagents

All herbicides analytical standards were supplied by Sigma-Aldrich (Inc. St. Louis, MO, USA). The individual stock standard solutions of 1000 mg L^{-1} were prepared in methanol by exact weighing of high-purity substances and stored at -18ºC in the dark. Then a mixture of all the compounds was prepared in methanol containing 10 mg L^{-1} each individual triazine and stored at -18ºC. All working solutions were daily prepared by appropriate dilution of the 10 mg L^{-1} standard solutions.

1-octanol Chromasolv® (grade HPLC 99%) was purchased from Sigma-Aldrich Co. (Madrid, Spain), methanol was superpurity solvent from Romil (Cambridge, UK) and acetonitrile was from Panreac (Barcelona, Spain). Ultrapure water was obtained using a Millipore Milli-Q system (Millipore, Bedford, MA, USA).

2.3. Apparatus

The HPLC-DAD system consisted of a 2695 pump with a 996 Diode Array Detector from Waters (Milford, MA, USA). The LC-ESI-MS/MS analyses were performed using an Agilent HP-1200 Series LC system equipped with an autosampler, a binary solvent pump and a thermostated column oven. The LC system is coupled to a mass spectrometer with a triple quadrupole detector (API 3200, Applied Biosystem, Carlsbad, CA, USA) equipped with an APCI/ESI source.

The column was a stainless steel column (150 mm x 4.6 mm ID, particle size 5 µm) packed with Hypersil GOLD C_{18} chemical bonded phase from Thermo Scientific (Austin, TX, USA).

2.4. Extraction procedure

Aliquots of 25 mL of filtered seawater samples were extracted as follows: 300 µL of 1-octanol was added as extractant solvent and the mixture was vigorously shaken using an agitation plate Vibrax-VXR by IKA (Staufen, Germany) during 10 min at 1200 rpm. The separation of both phases was performed by centrifugation (Eppendorf 5804, Madrid, Spain) at 3500 rpm for 3 min. The droplets of 1-octanol were collected and the

volume was adjusted to 1 mL with methanol due to the immiscibility of the 1-octanol with the mobile phase of liquid chromatography. To remove any solid particles that might interfere in the analysis and damage the equipment, the extract was passed through a 0.2 µm syringe filter of PTFE (Teknokroma, Barcelona, Spain) before LC injection. The schematized DLLME procedure is showed in Fig.2.

2.5. Chromatographic analysis

The HPLC-DAD analysis was carried out using the following acetonitrile:H_2O gradient elution: acetonitrile initial percentage of 30% (8 min), increased linearly to 40% in 5 min; increased to 50% in 5 min, after which the percentage was returned to the initial conditions in 9 min. A constant mobile phase flow rate of 1 mL min^{-1} and 20 µL of sample volume were used. The absorbance was measured continuously in the 200-400 nm range and peaks areas quantification were carried out at 222.7 nm in order to achieve maximum sensitivity. All triazine herbicides were identified initially by retention time and then by applying spectral contrast techniques (incorporated in Millenium[32] software) the homogeneity of the spectral peak was confirmed. Finally a spectral identification was carried out contrasting the spectrum with a standard library created in wavelength interval of 200-400nm.

The LC-ESI-MS/MS analysis of the herbicides was performed in the positive ionization mode. Data acquisition was performed in the multiple reaction monitoring mode, recording the transition between the precursor ion and the two most abundant product ions for each target analyte listed in Table 1.

3. Results and discussion

2.1. DLLME optimisation

The optimisation of the DLLME procedure was carried out by using seawater sample spiked with 10 µg L^{-1} of each triazine and three replicates were used for all assays. Several considerations must be taken into account to select an adequate extractant solvent in DLLME. The application is intended for a group of triazines in seawater matrices; therefore, the solvent must be immiscible with water. Several organic solvent (200 µL) were initially selected to develop the DLLME application: 1-octanol, dichloromethane and hexane. The organic solvent tends to form a single drop when it is added to a water sample; therefore, an agitation step (10 min, 1200 rpm) was needed to break down the drop of organic solvent and improve the dispersion process. The mixture was then centrifuged (3 min at 3500 rpm) in order to separate the phases. After centrifugation, it was observed that the hexane was not able to form the drop; while with the other solvents a drop was formed at bottom (dichloromethane) or top (1-octanol) of the test tube. Dichloromethane was discarded because the drop obtained is

more difficult to collect and furthermore, according to principles of green chemistry, for being a chlorinated solvent. Consequently, 1-octanol was the selected solvent.

The effect of extraction time on the extraction efficiency in DLLME method was studied over the range of 3-60 min. For this purpose, 30 mL of a seawater sample solution containing 10 µg L^{-1} of each triazine were extracted using 200 µL of 1-octanol and 1200 rpm. The results (see Fig. 3a) showed that the extraction efficiency slightly increased up to 10 min, kept constant until 40 min and a small decrease was achieved when the extraction time is too long (60 min). Therefore, 10 min was selected as the optimum extraction time.

The effect of the extraction solvent volume was investigated using different volumes of 1-octanol (100, 200 and 300 µL) keeping constant the other experimental conditions. The results obtained (see Fig. 3b) showed that the extraction efficiency increased with solvent volume being the recoveries obtained with 300 µL of 1-octanol higher than 90% for all compounds except for simazine (67%), cyanazine (72%) and symetrin (77%). Consequently, 300 µL was chosen as optimum extraction solvent volume. With a view to improving the recovery of simazine, cyanazine and symetrin, a study using 25 mL of seawater sample solution containing 10 µg L^{-1} of each triazine was carried out. The recoveries obtained were satisfactory for all triazines (> 80%).

To evaluate the effect of stirring rate, 25 mL of a seawater sample solution containing 10 µg L^{-1} of each triazine were extracted for 10 min with 300 µL of 1-octanol. The stirring rate varied in the range of 800-1400 rpm. As expected, a high stirring rate resulted in greater extraction efficiency obtaining the best results at 1400 rpm (see Fig. 3c). However, because high stirring rate could break the tube and the signal variation for simazine and cyanazine between 1200 and 1400 rpm is very small, 1200 rpm was selected as stirring rate for sample extraction.

The influence of the matrix on the peak area of the compounds was evaluated since sensitivity, reproducibility and accuracy can be greatly affected. The matrix effect was studied by comparison of the slopes of the calibration curves in methanol-octanol (7:3) and in the extract obtained after the DLLME procedure. The results showed that there are not slope deviation between matrix calibration and solvent calibration.

Furthermore, it is known that the addition of NaCl into the sample solution leads to increase ionic strength of sample solution and enhanced the extraction efficiency of triazine herbicides. Several authors have pointed out this salting-out effect on the extraction of triazine herbicides. So, Sanagi et al. (2012) developed a DLLME method for determination of simazine, atrazine and cyanazine in lake and tap water adding NaCl 5% (w/v). Ye et al. (2007) employed 15% (w/v) of NaCl to determine simazine, atrazine and cyanazine by single-drop microextraction in tap and reservoir waters and Wang et

al. (2011) used 10% (w/v) of NaCl to analyze simazine, atrazine and ametryn in reservoir, river and well water. In this work, it was not necessary to study the influence of salt addition because the matrix studied was seawater.

On the other hand, since the triazines are ionisable compounds, the pH of sample solution can also affect the extraction efficiency. So, Ye et al (2007) studied the effect of the pH on the extraction efficiency of triazines in water samples and reported that highest recoveries were achieved at the pH range of 5-9. Therefore during experimental development the pH of the samples was not adjusted because seawater shows a pH at the range 7.5-8.4.

3.2. Method validation

The method was validated in terms of linearity, recoveries, precision and limits of detection and quantification. All quantitative results were calculated using 25 mL of unpolluted seawater sample spiked with a standard mixture of the compounds. The results obtained are presented in Table 2.

The linearity of the matrix calibration curves was evaluated by triplicate analysis at six different concentration levels (1, 2, 3, 4, 5 and 6 µg L^{-1}). The calibration data obtained in this study are shown in Table 2. As it can be seen, very good linearity was obtained for all compounds with $r^2 > 0.999$, except for simazine which showed a determination coefficient only slightly lower ($r^2 = 0.9975$). The limits of detection (LODs) were determined as $3*S_{y/x}/b$ and the limits of quantification (LOQs) as $10*S_{y/x}/b$, where $S_{y/x}$ is the residual standard deviation and b is the slope of the matrix calibration curves. As it can be seen in Table 2, the detection and quantification limits obtained (between 0.06-0.37 and 0.19-1.12 µg L^{-1} respectively) were adequate, being the LOQs much lower than 30% of the parametric value requested by the legislation for surface water, except for simazine which showing about half of the legislated value (Council of the European Communities 2008a).

By comparing the LODs obtained by the proposed method with those reported in the literature using DLLME for the extraction of triazines in water, a better sensitivity is attained respect to Wang et al. (2010), who studied the limits of detection employing deionised water instead of water sample. Zhou et al. (2009) have obtained better and worse LODs for simazine and atrazine respectively using deionised water instead of a water sample. Wang et al. (2011) studied the extraction of triazines in river, reservoir and well water and the LODs obtained are better for simazine and ametryn but worse for atrazine. It should be noted that the calculation of LODs was based on a signal-to-noise ratio (S/N) of 3. On the other hand, Nagaraju et al. (2007) and Sanagi et al. (2012) have shown better LODs for the three triazine herbicides studied in each case (except

for atrazine in the first case), however it is important to take into account that the analysis was performed by gas chromatography coupled to mass spectrometry.

Repeatability, intermediate precision and accuracy were investigated by analysis of unpolluted seawater samples spiked at 2 µg L^{-1}. The spiked concentration was selected to test the method performance at the residue level set by European Union Legislation for atrazine in surface waters (Council of the European Communities 2008a). The repeatability was calculated as within-day RSD of peak areas using ten replicates analyzed in the same day and by the same analyst. In the case of intermediate precision five replicates were analyzed in consecutive days and by the same analyst and it was calculated as between-day RSD of peak areas. As it can be seen in Table 2, the results obtained were satisfactory with RSD values below 5% and 10% for repeatability and intermediate precision respectively.

Accuracy of the method was evaluated in terms of recovery. The results obtained for five replicates of the sample spiked with the triazine herbicides are presented in Table 2. The results demonstrated that the method achieved satisfactory recoveries in the range of 81-102%, with associate standard deviations below 3% for all compounds. Furthermore, the obtained recoveries and relative standard deviation are comparable (Wang et al. 2011; Sanagi et al. 2012) or even much better (Zhou et al. 2009; Wang et al. 2010; Nagaraju et al. 2007) to those provided by other authors for the determination of some of these pollutants by DLLME in waters.

The advantages of this method when compared with other ones described in the bibliography are: 1) The use of agitation step for dispersion instead of a disperser solvent avoids the main DLLME problem that is the adequate selection of the mixture of solvent/dispersant because analytes losses can occur; 2) In this study seven triazines are determined whereas in other studies using DLLME only between two and five triazines are analyzed, except Nagaraju et al. that determine eight triazines but only four of them match up with triazines of the proposed method (simazine, atrazine, propazine and simetryn) and 3) The method is validated for seawater while other authors applied their methods to other types of surface waters (farm, lake, groundwater, river and reservoir waters) or tap water (Nagaraju et al. 2007; Zhou et al. 2009; Wang et al. 2010; Wang et al. 2011; Sanagi et al. 2012). Therefore, it can be concluded that the proposed method could be a competitive analytical tool for the analysis of triazines at trace levels in seawater samples in compliance with EU directives. As an example, Figure 4 shows the chromatograms corresponding to unspiked and spiked seawater sample (2 µg L^{-1}) extracted using the DLLME method studied.

3.3. Application of the method to the analysis of estuarine seawater samples

The proposed method was applied to analyse the target compounds in ten water samples from the estuary of A Coruña (Galicia, NW of Spain). The sampling took place during March of 2013. The analysis showed that the concentration of the triazines were below the LODs.

4. Conclusions

A fast and simple method for the analysis of seven triazines from seawater samples has been developed. The method is based on DLLME prior to HPLC-DAD identification and quantification. Confirmation with LC-ESI-MS/MS was taken into account.

The method uses small volumes of organic solvent in agreement with the principles of the Green Chemistry and agitation instead of dispersive solvent which simplifies the experimental procedure.

The developed method has shown suitable precision and accuracy for determination of triazines in seawater samples. The limits of quantification enable the determination of these pollutants at the levels required by European Union legislation (Council of the European Communities 2008a) using only 25 mL of sample; consequently it can be an important tool to control the presence of triazines in estuarine seawater samples. Furthermore, it is important to note that to the best of our knowledge no previous studies have been published to the determination of these seven triazines in seawater.

The proposed method was applied to determine the target compounds in seawater from estuary of A Coruña (Galicia, NW of Spain). Although the triazines under study have not been detected in the samples analyzed, the monitoring of their levels in marine ecosystems is of great economic and environmental importance. It is important to take into account that measurements of known quality represent the foundation of the water quality evaluation system and the basis for decisions to be taken to achieve Marine Strategy Framework Directive and environmental objectives at the end of 2015 (Council of the European Communities 2008b).

Acknowledgements

The authors acknowledge the financial support of the “Ministerio de Educación y Ciencia” (Project CTQ2007-63949/BQU) and the “Xunta de Galicia” (Project 09MDS038103PR and “Ayuda Consolidación e Estructuración de Unidades de Investigación Competitivas”). N. Rodríguez-González also thanks the “Xunta de Galicia” for the concession of her award under the program “Campus del Mar”.

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Figure 1. Location of sampling sites in estuary of A Coruña (Galicia, NW of Spain)

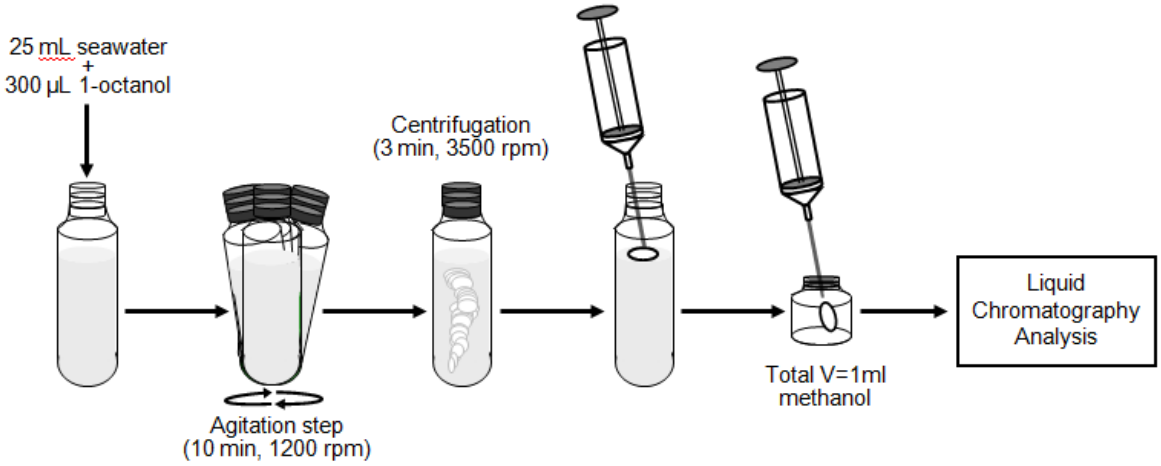
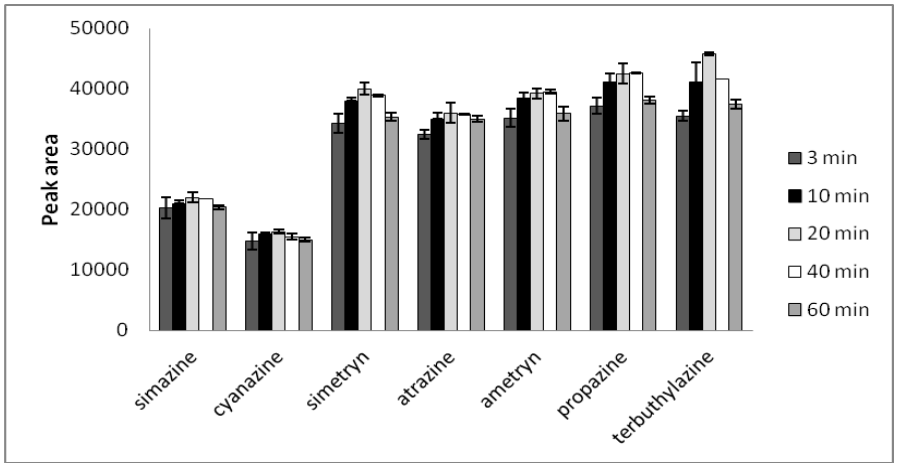
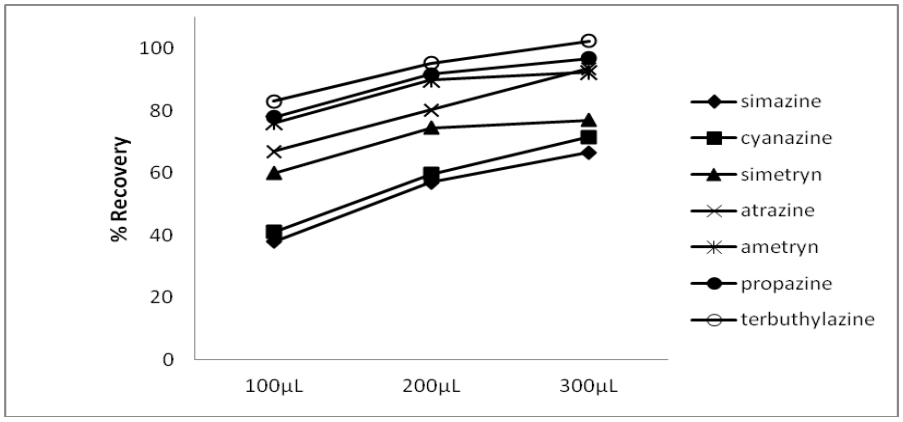


Figure 2. Scheme of DLLME procedure

(a)



(b)



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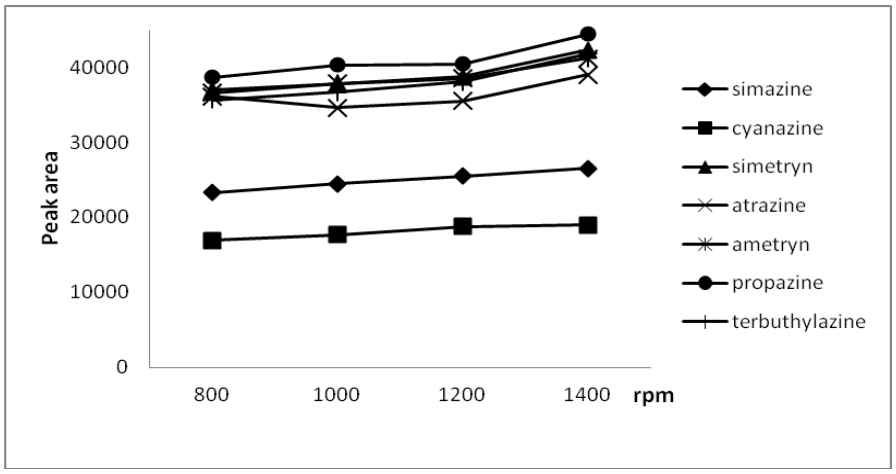
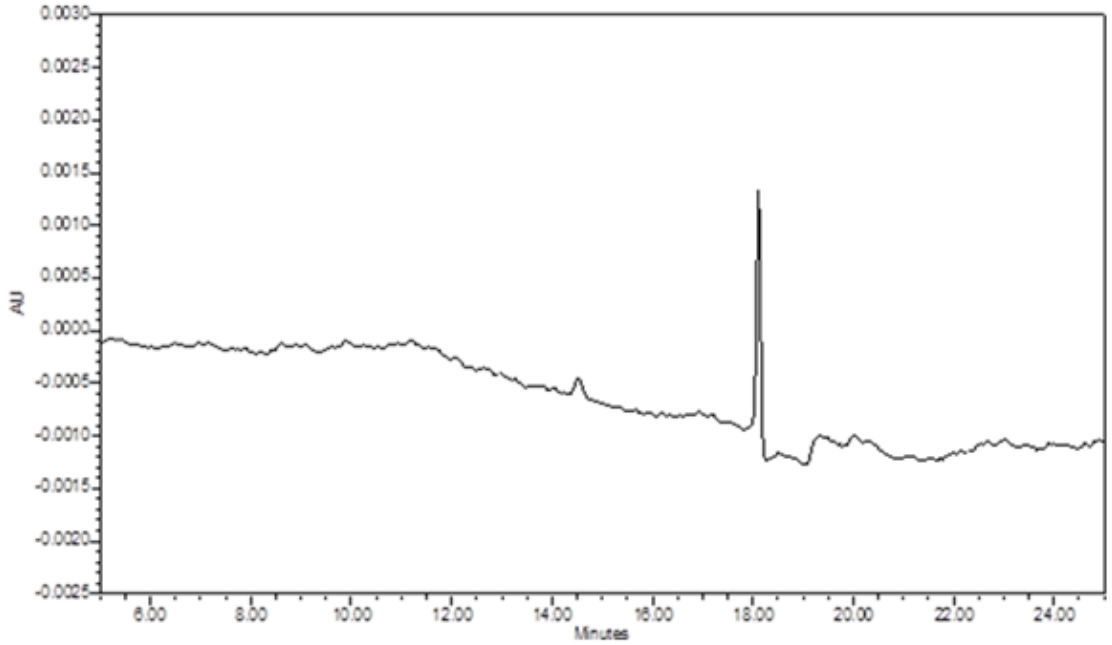


Figure 3. (a) Effect of extraction time on the extraction efficiency in DLLME (n = 3); (b) Effect of extraction solvent volume in DLLME (n = 3) and (c) Effect of stirring rate on the signal (n = 3)

(a)



(b)

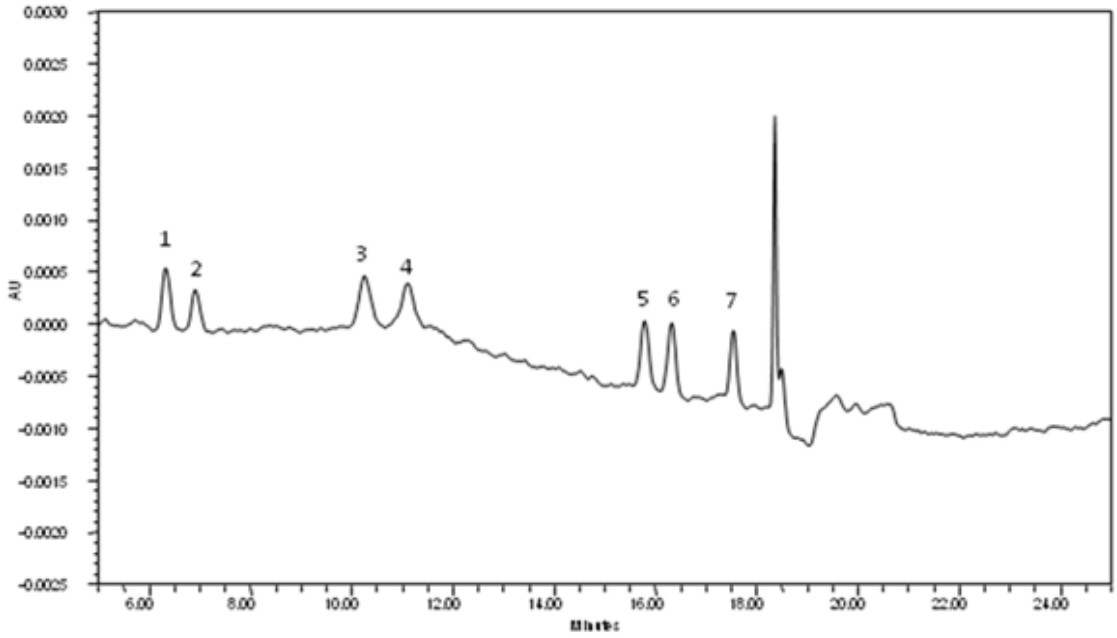


Figure 4. Chromatograms obtained after DLLME (a) seawater sample, (b) seawater sample spiked at 2 µg L^{-1}. Target compounds are numbered as follows: (1) simazine, (2) cyanazine, (3) simetryn, (4) atrazine, (5) ametryn, (6) propazine, (7) Terbuthylazine

Table 1. Optimized conditions for LC-ESI-MS/MS analysis of the investigated herbicides

Analyte	t_R (min)	Transitions (*m/z*) Precursor ion →product ion	Declustering potential (V)	Collision energy (V)
Simazine	6.62	202 → 104	41	33
		202 → 68	41	45
Cyanazine	7.23	241 → 214	46	21
		241 → 68	46	53
Simetryn	9.48	214 → 68	41	47
		214 → 124	41	29
Atrazine	9.94	216 → 174	41	23
		216 → 68	41	49
Ametryn	13.31	228 → 186	41	23
		228 → 68	41	51
Propazine	13.70	230 → 146	41	33
		230 → 58	41	37
Terbuthylazine	14.82	230 → 174	36	21
		230 → 68	36	49

Table 2. Analytical characteristics of the DLLME-HPLC-DAD method

Compound	LOD (μg L^{-1})	LOQ (μg L^{-1})	Determination coefficient (R^2)	[a] Intercept interval	[a] Slope interval	[a] CV Slope	Repeatability [b] RSD (%)	Reproducibility [b] RSD (%)	Recovery (%)
Simazine	0.37	1.10	0.9975	604 ± 0.34	2086 ± 0.09	0.0044	1.8	3.9	81 ± 0.4
Cyanazine	0.13	0.40	0.9996	69 ± 0.11	1397 ± 0.03	0.0019	1.8	8.0	81 ± 0.6
Simetryn	0.11	0.33	0.9997	-9 ± 0.09	3242 ± 0.02	0.0007	2.2	5.4	86 ± 2.7
Atrazine	0.06	0.19	0.9999	-2 ± 0.05	3422 ± 0.01	0.0004	2.0	8.6	86 ± 2.0
Ametryn	0.09	0.27	0.9998	-161 ± 0.08	3082 ± 0.02	0.0006	4.1	5.8	96 ± 0.8
Propazine	0.15	0.47	0.9995	-17 ± 0.13	3188 ± 0.03	0.0010	4.5	6.4	94 ± 2.0
Terbuthylazine	0.14	0.43	0.9996	-134 ± 0.12	2968 ± 0.03	0.0010	2.6	8.3	102 ± 2.5

[a] Confidence level 95%

[b] n = 10 and n = 5 for repeatability and reproducibility respectively (2 μg L^{-1})

ON-LINE SOLID-PHASE EXTRACTION METHOD FOR DETERMINATION OF TRIAZINE HERBICIDES AND DEGRADATION PRODUCTS IN SEAWATER BY ULTRA-PRESSURE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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ABSTRACT

A fast, simple, selective and sensitive method has been developed for the determination of nine triazine herbicides (ametryn, atrazine, cyanazine, prometryn, propazine, simazine, simetryn, terbuthylazine and terbutryn) and eight degradation products (desethyl atrazine, desethyl-desisopropyl atrazine, desethyl 2-hydroxyatrazine, desethyl terbuthylazine, desisopropyl atrazine, desisopropyl 2-hydroxyatrazine, 2-hydroxyatrazine and 2-hidroxyterbuthylazine) in seawater samples. On-line solid-phase extraction coupled with ultra-pressure liquid chromatography-tandem mass spectrometry was employed for simultaneous analysis of all compounds in 11 min. Validation parameters were studied through the estimation of the limits of detection and quantification, calibration curves and precision. Limits of quantification ranged from 0.023 to 0.657 μg L^{-1}. Good linearity was obtained for all compounds with $R^2 > 0.99$ in all cases. Furthermore, inter-day precision (0-2.1%) and intra-day precision (0-3.9%) were shown to be satisfactory. On-line solid-phase extraction recoveries in spiked unpolluted seawater sample were evaluated and acceptable values (80.3-99.8%) with adequate RSD (0.1-3.1%) were found.

Finally, the proposed method was applied to the analysis of the target compounds in seawater samples collected from seawater nearby a zone of intensive horticulture of Matosinhos (Portugal). The concentrations of the herbicides were below the limit of detection in all cases.

Keywords: Triazines; Degradation products; Seawater; Ultra-pressure liquid chromatography; Mass spectrometry

1. Introduction

The occurrence of micropollutants has been highlighted in thousands of publications during the last decade, which have pointed out a growing concern about them. Although there are no discharge limits for most micropollutants, some regulations have been published. Many of these substances, including triazine herbicides, are toxic and hazardous; as an example, atrazine produces genotoxic damage in fish species [1]. Therefore, the chemical pollution of surface water can affect the environment, several effects as chronic toxicity on aquatics organisms, accumulation in the ecosystem, as well as injures in human health are described. The European Union has included simazine and atrazine in the list of 33 priority substances in the EU Water Framework Directive (2000/60/EC) by way of Decision 2455/2001/EC [2]. Moreover, the Directive 2008/105/EC [3] sets the Environmental Quality Standards (EQS) for these compounds in surface water. Recently, the Directive 2013/39/EU amending the Directives 2000/60/EC and 2008/105/EC includes terbutryn to the list of priority substances [4]. This Directive establishes a maximum permitted concentration of 0.34, 2 and 4 μg L^{-1} for terbutryn, atrazine and simazine respectively. Furthermore, Directive 2013/39/EU calls the attention on the important role of monitoring emerging pollutants that are not regularly considered in monitoring programs but can have toxicological effects.

In this way, studies examining the concentration of triazines in surface waters have expanded the list of compounds including their main degradation products [5-7]. These degradation products are produced through abiotic and biotic processes in the soil, groundwater and surface water [8]. Because of their mobility in the soil-water environment, the degradation products can reach water bodies more easily than triazines; thus, the impact due to herbicides tends to be underestimated when only the triazines are analysed in samples. Therefore, the main degradation products should be included in current analytical methods to obtain a better knowledge of water quality regarding herbicides contamination [9].

In order to support the implementation of the Directive 2013/39/EU, simple, selective and sensitive analytical methods are required. Different chromatographic techniques have been used to determine triazines and/or their degradation products in water. Between these techniques, the application of liquid chromatography-tandem mass spectrometry (LC-MS/MS) has provided an increased selectivity and sensitivity [10-16]. The recent development of chromatographic columns using new stationary phases with particle size < 2 μm for ultra-pressure liquid chromatography (UPLC) allows significantly shorten the analysis time [17]. UPLC-MS/MS can offer not only good sensitivity but also high confidence in the confirmation of compounds detected allowing to achieve more than 3-4 identification points. Thus, UPLC coupled to tandem MS

provides an interesting tool for fast determination of these compounds in water samples [18-21].

Regarding extraction procedure, off-line SPE methods usually require high sample volume and are time-consuming [22]. On the other hand, although on-line SPE provides advantages, it also has disadvantages including the complexity of the set-ups of valve-switching, lack of flexibility as compared to off-line SPE and possible interferences from loading the entire extracted sample. Most of these problems have been resolved with advances in automated on-line SPE systems, integrated and flexible software programs and application of tandem spectrometry detectors for better selectivity. On-line SPE offers advantages, such as the minimal amount of solvents required for extraction, fast sample preparation and small sample volumes [23]. For this reason, the use of on-line SPE coupled to liquid chromatography-mass spectrometry has increased and some methods have been published for the analysis of triazines and their degradation products in water [7, 13, 20]. Nonpolar SPE sorbents are generally selected for extracting triazines from water samples. However, the degradation products, which contain polar functional groups, can be more efficiently extracted by using polar sorbents [24]. For SPE, different solid phases such as Oasis MCX cartridges [25, 26], PLRP-s [13, 20], Oasis HLB cartridges [15, 20], Amberlite XAD-4 resin [27] and Oasis MAX cartridges [16] have been employed for triazines and their major hydroxy and dealkylated products. Several articles have compared different adsorbents and Oasis HLB has shown to have better ability to retain both non-polar and polar compounds.

The aim of this study was to develop a fast, simple, selective and sensitive analytical method for the quantification and confirmation of 17 compounds (nine triazine herbicides as well as their main degradation products) in seawater. The method developed is based on an on-line solid-phase extraction (OASIS HLB cartridges) followed by UPLC-MS/MS determination. This method enables the determination of these pollutants at the levels required by European Union legislation [4]; consequently, it can be an important tool to control the presence of triazines and their degradation products in seawater samples. To the best of our knowledge, studies using on-line SPE-UPLC-MS/MS have not been done to determine the target compounds in seawater. However, the monitoring of these compounds in marine ecosystems situated close to areas of intensive horticulture is of great interest. Finally, the proposed method was applied to determine the target herbicides in seawater samples from beaches of Matosinhos (Portugal).

2. Experimental

2.1. Study area and sampling

The studied area is situated in the Portuguese seashore. This area presents some vulnerability due to hydrogeological factors and the interdependence with properties of pesticides what may add higher importance to the groundwater contamination and in this particular case reaching seawater [28]. Figure 1 shows the study area with the sampling points. Seawater samples were collected from ten beaches susceptible to contamination by triazines during December of 2015. Sampling points are listed below: 1 (Angeiras Norte), 2 (Angeiras Sul), 3 (Fontão), 4 (Pedras Brancas), 5 (Pedras do Corgo), 6 (Agudela), 7 (Marreco), 8 (Memória) 9 (Cabo do Mundo) and 10 (Aterro).

Seawater samples were collected in amber glass bottles and transported to the laboratory under cooled conditions (4ºC). At each sampling point, three samples were collected. Upon reception, samples were filtered through a PTFE syringe filter to eliminate suspended solid matter. Due to the low stability of triazines, the samples were analysed the day of sampling.

Furthermore, unpolluted seawater samples from the Circunvalação beach at the city of Porto were used for method validation.

2.2. Chemicals and materials

All herbicides analytical standards (purity between 97.5 and 99.5) were supplied by Sigma-Aldrich (Inc. St. Louis, MO, USA). The structures of the target compounds are shown in Figure 2. The individual stock standard solutions of 1000 mg L^{-1} were prepared in methanol by exact weighing of high-purity substances and stored at -18ºC.

Mixed stock solution of 17 compounds was prepared in methanol containing 1 mg L^{-1} for desethyl-desisopropyl atrazine (DEDIA), desethyl 2-hydroxyatrazine (DEHA), desisopropyl atrazine (DIA), desisopropyl 2-hydroxyatrazine (DIHA) and 2-hydroxyterbuthylazine (HT); and 0.1 mg L^{-1} for the nine triazines, desethyl atrazine (DEA), desethyl terbuthylazine (DET) and 2-hydroxyatrazine (HA). All working solutions were daily prepared by appropriate dilution of the mixed solution with methanol.

Methanol for liquid chromatography was supplied by J.T. Baker (Deventer, the Netherlands), and Milli-Q water was obtained from a purification system from Millipore (Molsheim, France). Ammonium acetate (≥ 99%) (LC grade) was purchased from Sigma-Aldrich (Inc. St Louis, MO, USA).

OASIS® HLB Direct connect HP columns 20 µm (2.1 x 30 mm) were supplied by Waters (Milford, MA, USA). PTFE syringe filters (0.2 µm pore size) were purchased from Merck (Darmstadt, Germany).

2.3. Ultra-pressure liquid chromatography

The pesticides and metabolites were separated by an Acquity system (Waters, Manchester, UK) consisting of an Acquity UPLC™ binary solvent manager, an Acquity UPLC™ sample manager and an Acquity UPLC™ heater equipped with an Acquity UPLC BEH C18 column (2.1 mm x 100 mm, 1.7 µm particle size) with a pre-column (UPLC BEH C18 2.1 mm x 5 mm, 1.7 µm particle size). These columns are packed with a C18 reverse-phase bounded to ethylene-bridged hybrid (BEH) substrate. Elution was performed under gradient mode using 5 mM ammonium acetate in water as mobile phase A and 5 mM ammonium acetate in methanol as mobile phase B. Separation was carried out at 40ºC using a flow rate of 300 µL min^{-1}. The gradient elution was performed as follows: mobile phase A initial percentage of 95% decreasing linearly to 0% in 5 min and holding in it for 3 min, after which the percentage was returned to the initial conditions in 0.1 min. Initial conditions were maintained during 3 min for the column re-equilibration. The sample volume injected into the UPLC system was 5 mL. The optimized method allowed the concurrent detection of 17 pesticides in a chromatographic run of 11 min.

2.4 Tandem mass spectrometry

An Acquity triple-quadrupole mass spectrometer (Acquity TQD™ Waters, Manchester, UK) fitted with an electrospray ionization source (ESI) was used for pesticide detection. MS/MS detection was performed in negative or positive mode, depending on the compounds. ESI mode was set individually for each target compound, the switch time between positive and negative mode was 20 ms. Data acquisition was performed under multiple reaction monitoring (MRM) mode, recording the transition between the precursor ion and the most abundant product ions for each target analyte. The optimization of MS/MS conditions, including the search for precursor and product ions, optimization of the sample cone voltage, collision energy was performed using an Intellistart® module of the MassLynx 4.1 software. MRM conditions used for the analysis of pesticides are shown in Table 1.

The ESI interface conditions were optimized for maximum intensity of the precursor ions as follows: capillary voltage 3.5 kV, source temperature 140 ºC and desolvation temperature 350 C. The nebulizer and desolvation gas flows were set at 850 and 50 Lh^{-1} respectively. Nitrogen was used as nebulizer and desolvation gas. Argon was used as collision gas at a pressure of 3.7 10^{-3} mBar. The analytical device was controlled by MassLynx software (version 4.1 SCN 714) and the data were processed using QuanOptimize software.

2.5 On-line solid phase extraction

The SPE was performed by a CTC hTC PAL autosampler (fitted with a 5 mL syringe, a 5 mL loop and a sample rack of 32 x 10 mL) coupled to the UPLC-MS/MS instrument. On-line SPE was carried out using a device, which employs a dual LC column system; one OASIS HLB column for pre-concentration and another for analytical separation (see Figure 3). The extraction of all seawater samples, aqueous standard solutions and blanks was performed by loading 5 mL of the solution through an OASIS HLB on-line SPE cartridge previously conditioned with water/methanol (95:5, v/v) during 10 min (flow rate of 1 mL min^{-1}). The trap cartridge was fitted into the load position A of a valve switching Rheodyne 10 port. A Waters isocratic pump was used to load the sample at 5 mL min^{-1} onto the trapping cartridge. After sample loading (11 min), the valve was switched to position B being the analytes eluted from the cartridge to the LC column with the chromatographic mobile phase using a Waters Binary pump.

3. Results and discussion

3.1. UPLC-MS/MS development

The optimum cone voltage and collision energy for each pesticide was selected with the aim of obtaining the precursor ion and the MRM transition with the highest sensitivity and the other product ions (see Table 1 and Figure 4). The most sensitive transition was chosen for quantification and the other transitions were used for confirmation. Optimization of MS/MS settings was performed by direct infusion of individual standard solutions (0.1-0.5 µg mL^{-1} in methanol). Negative or positive mode was studied for all analytes. The analysis of cyanazine was performed in the negative mode and the determination of the remaining compounds was performed in the positive mode. The manufacturer recommended values for desolvation and cone gas flow were selected. The suitable setting of the instrument for each pesticide is shown in Table 1.

Once MS parameters were established, the UPLC conditions were optimized in order to attain an adequate elution of the target compounds and a short analysis time. The mobile phase composition was evaluated considering that chromatographic behaviour of some compounds was very similar and their separation was difficult. Furthermore, degradation products are strongly dependent on pH and the use of a mobile phase with a buffer or a modifier is necessary. For this reason, different mobile phases (water and methanol with and without ammonium acetate) were tested and the best chromatographic separation was achieved by using 5 mM ammonium acetate in both mobile phases. In view of the satisfactory results obtained, other modifiers as formic acid or acetic acid were not tested.

Various analytical gradients were also evaluated. Using the conditions described in section 2.3, all the compounds were adequately separated within a run time of 11 min.

The chromatograms obtained from a spiked seawater sample with 0.1 µg L^{-1} (the nine triazines, DEA, DET and HA) and 1 µg L^{-1} (DEDIA, DEHA, DIA, DIHA and HT) are shown in Figure 5.

3.2. Method performance

The analytical method is based on a method previously developed by the authors for the analysis of the nine target triazines in seawater using Oasis HLB by means of off-line SPE [29]. Furthermore, it was taken into account that Oasis HLB has shown to have better ability to retain some degradation products than other sorbents. Thus, Gervais et al [18] studied different sorbent for multiresidue determination of pesticides in water including atrazine, cyanazine, DEA, DIA, simazine and terbuthylazine and the best recoveries were obtained by the extraction with Oasis HLB. Furthermore, Benvenuto et al. [19] have tested two sorbents Oasis HLB and Oasis MCX for the determination of atrazine, simazine, terbuthylazine, terbumeton, terbutryn and some of their main degradation products (DEA, DIA, DET, HA, HT) and Oasis HLB showed better ability to retain both non-polar and polar compounds.

Since the initial conditions assayed (described in section 2.5) have shown to be adequate, more studies about parameters such as sample volume, sample loading flow and elution were considered unnecessary.

The method was validated by estimation of the linearity, trueness and precision, limits of detection and limits of quantification. The results obtained are presented in Table 2.

The linearity was evaluated using standard solutions analysed with the method proposed. Calibration curves were constructed using seven calibration points, with three replicates for each calibration level, at the concentration range shown in Table 2. As it can be seen, very good linearity was obtained for all compounds with coefficients of determination (R^2) higher than 0.999, except for DIHA and HA which showed a coefficient of determination only slightly lower (0.9987 and 0.9989 for DIHA and HA respectively).

The limits of detection (LODs) were calculated as $3*S_{y/x}/b$ and the limits of quantification (LOQs) as $10*S_{y/x}/b$, where $S_{y/x}$ is the residual standard deviation and b is the slope of the matrix calibration curves. As it can be seen in Table 2, the limits of detection and quantification obtained (between 0.008-0.020 and 0.023-0.061 µg L^{-1} respectively) for triazine herbicides and three degradation products (DEA, DET and HA) were satisfactory. LOQs are much lower than 30% of the more restrictive parametric value requested by the legislation for triazines in surface water (maximum permitted concentration of 0.34 for terbutryn).

For the remaining degradation products, the limits of detection and quantification (between 0.133-0.217 and 0.402-0.657 µg L^{-1} respectively) were adequate, being the LOQs ≤ 30% of the parametric value requested by the legislation for atrazine in surface water [4]. Moreover, the selectivity of the method was evaluated by analysing control blank samples. The absence of any signal at the same retention time of the selected compounds indicated there were no any matrix interferences or contamination that can give a false positive signal.

The precision of the overall analytical procedure, expressed as relative standard deviation (RSD), was evaluated as intra-day and inter-day precision. To study intra-day precision, RSDs were calculated at two levels of concentrations measuring ten replicates at each concentration level during the same day. As it can be seen in Table 2, the obtained values were satisfactory for all compounds (RSDs lower than 4%). The inter-day precision was studied at one level of concentration by measuring three replicates on five consecutive days. The obtained values lower than or equal to 2.1% (see Table 2) indicate that the developed method was reproducible.

Trueness of the method was determined by spiking 5 mL of seawater sample at two concentration levels depending on the compound (0.1 µg L^{-1} for the nine triazines, DEA, DET and HA and 1 µg L^{-1} for DEDIA, DEHA, DIA, DIHA and HT).

To evaluate the trueness, the analytical recoveries of spiked samples, using five replicates at each fortification level, were calculated. As it can be seen in Table 2, satisfactory recoveries for all compounds in the range of 80.3-99.8% were achieved with associate standard deviations below 3.1.

3.3. Application of the method to the analysis of seawater samples

The proposed method was applied to analyse the target compounds in seawater samples of ten beaches susceptible to contamination by triazines in the seashore of Matosinhos (North of Portugal). Although none of the seawater samples contained detectable amount of target triazines and degradation products, this study is part of a project in which a detailed study with sampling in spring, summer and early autumn could take into account. During these months, it is time of sowing common crops and mainly fumigating different crops and vineyards.

4. Conclusions

On-line SPE-UPLC-MS/MS method has shown to be a fast, sensitive and robust alternative method to traditional off-line SPE for the analysis of nine triazines and their main degradation products in seawater samples. The proposed method is suitable to be used in routine analysis due to sample pre-treatment is not required and allows rapid trace enrichment from low sample volume with minimal sample handling. Furthermore,

on-line SPE reduces chemical waste due to the use of a minimal amount of extraction solvents. The method has shown suitable precision and good recovery values were obtained for all compounds.

The limits of quantification enable the determination of these pollutants at the levels required by European Union legislation [4] using only 5 mL of sample. Consequently, it can be an important tool to control the presence of triazines and their degradation products at trace levels in seawater samples in compliance with EU directives. It is noteworthy that a few methods based on solid-phase extraction combined with UPLC-MS/MS have been used to measure triazines and degradation products in river waters; however, there are not studies in seawater. Furthermore, an important difference of the proposed method with previously described methodology for the analysis of triazines herbicides and their main degradation products is the determination of a greater number of degradation products simultaneously with triazines.

The method was applied to the analysis of the target compounds in seawater from the coastline of Matosinhos (North of Portugal). Although the triazines and degradation products under study have not been detected in the samples analysed, the monitoring of their levels in marine ecosystems situated close to areas of intensive horticulture is of great local interest both economic and environmental.

Acknowledgements

N. Rodríguez-González thanks the "Xunta de Galicia" and Campus do Mar (International Campus of ExcellenceCompetitividad (CTM2013-48194-C3-2-R) and Program of Consolidation and Structuring of Units of Competitive Investigation of the University System of Galicia (Xunta de Galicia) potentially cofounded by European Regional Development Fund (ERDF) in the frame of the) for the concession of her PhD. Grant and the Universidade da Coruña for the concession of her INDITEX-UDC 2015 Grant for her stay in Porto.

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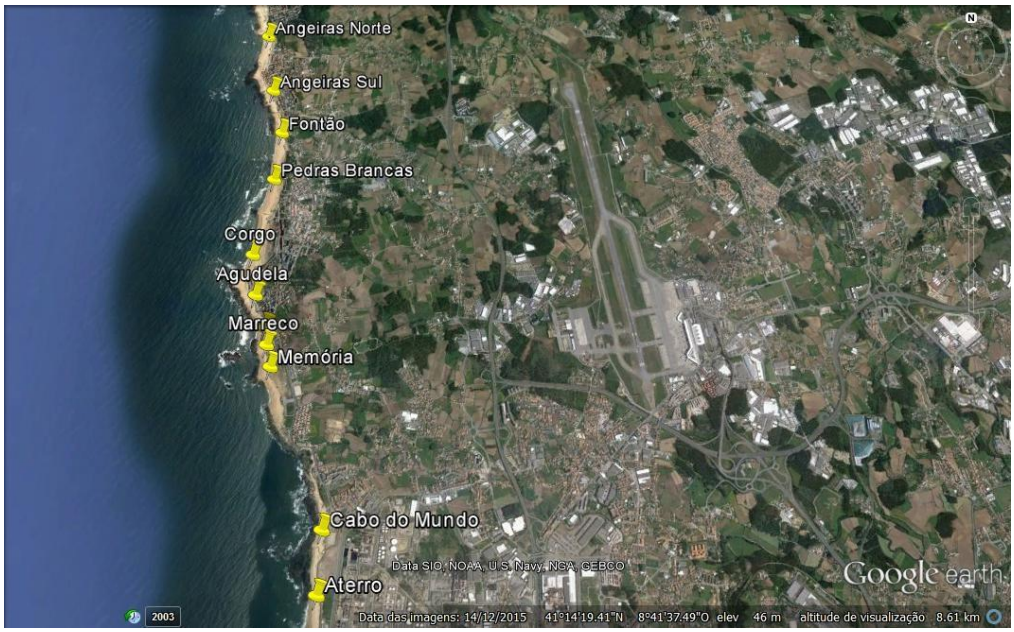

Figure 1. Location of sampling sites in Matosinhos (North of Portugal)

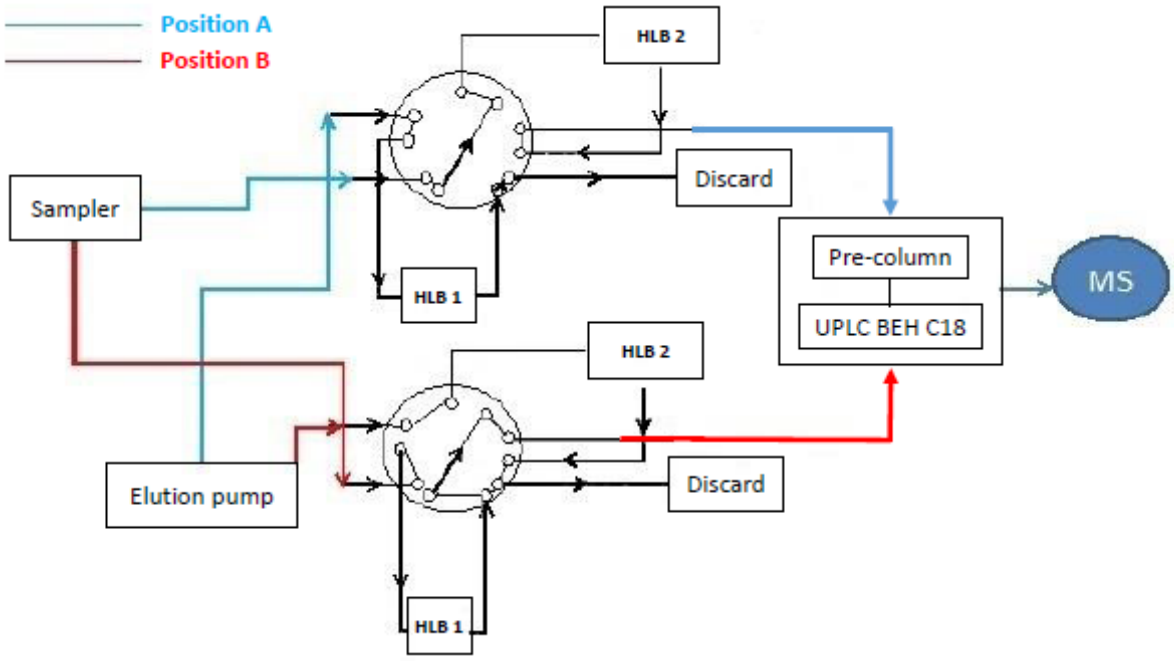

Figure 3. Scheme of the on-line SPE-UPLC-MS/MS

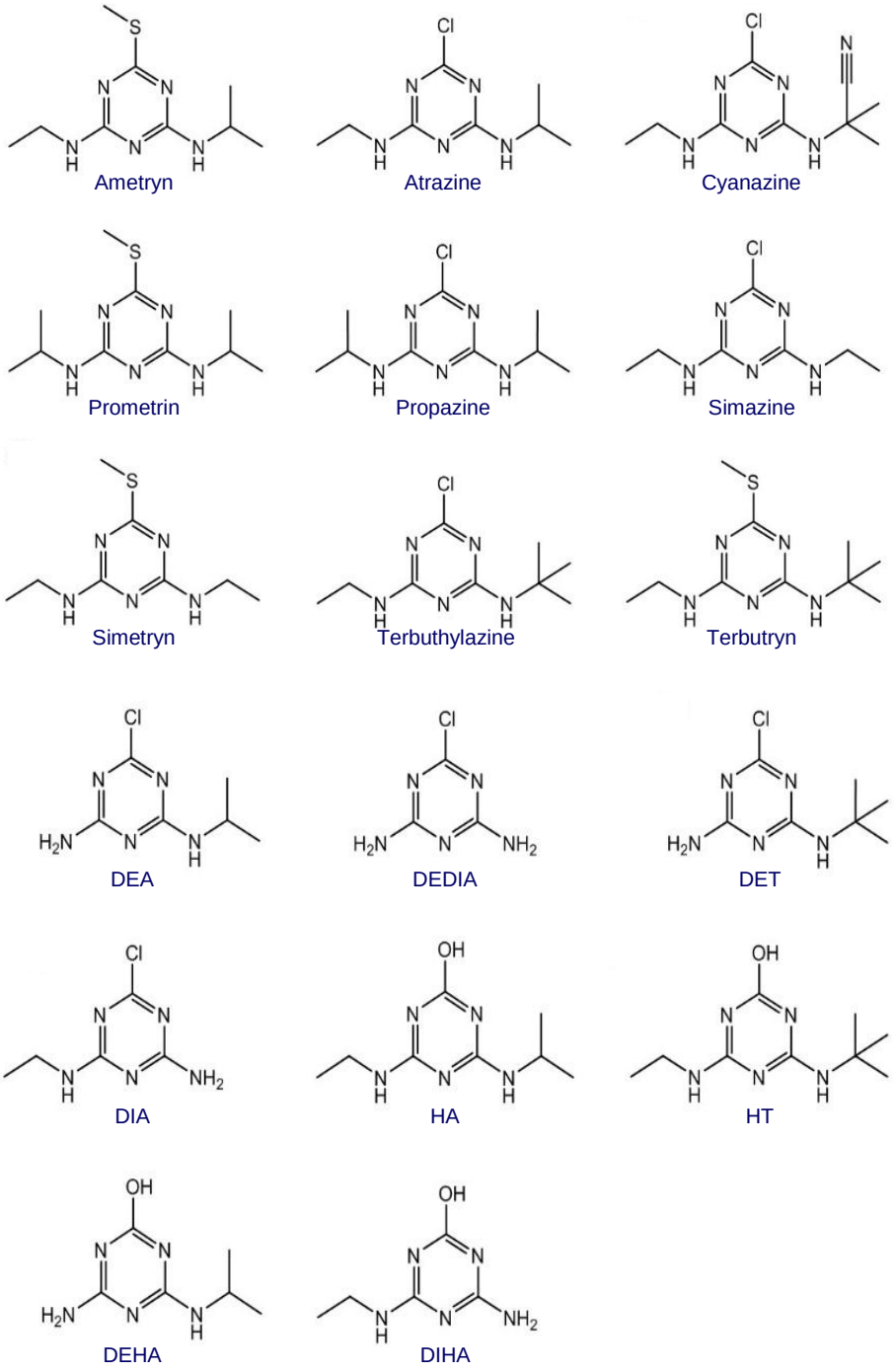

Figure 2. Structures of the target compounds

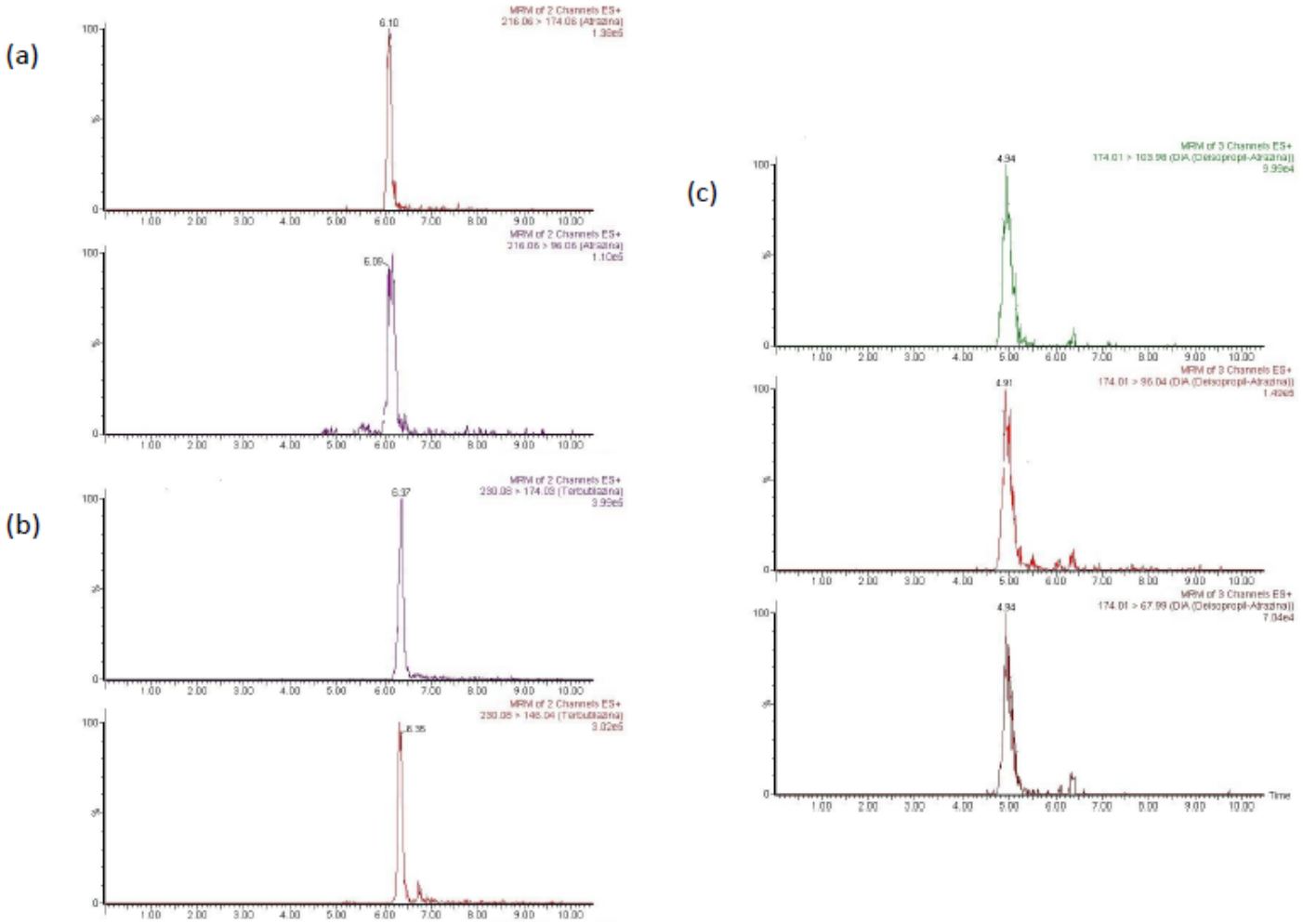

Figure 4. MRM transitions (a) atrazine, (b) terbuthylazine and (c) DIA

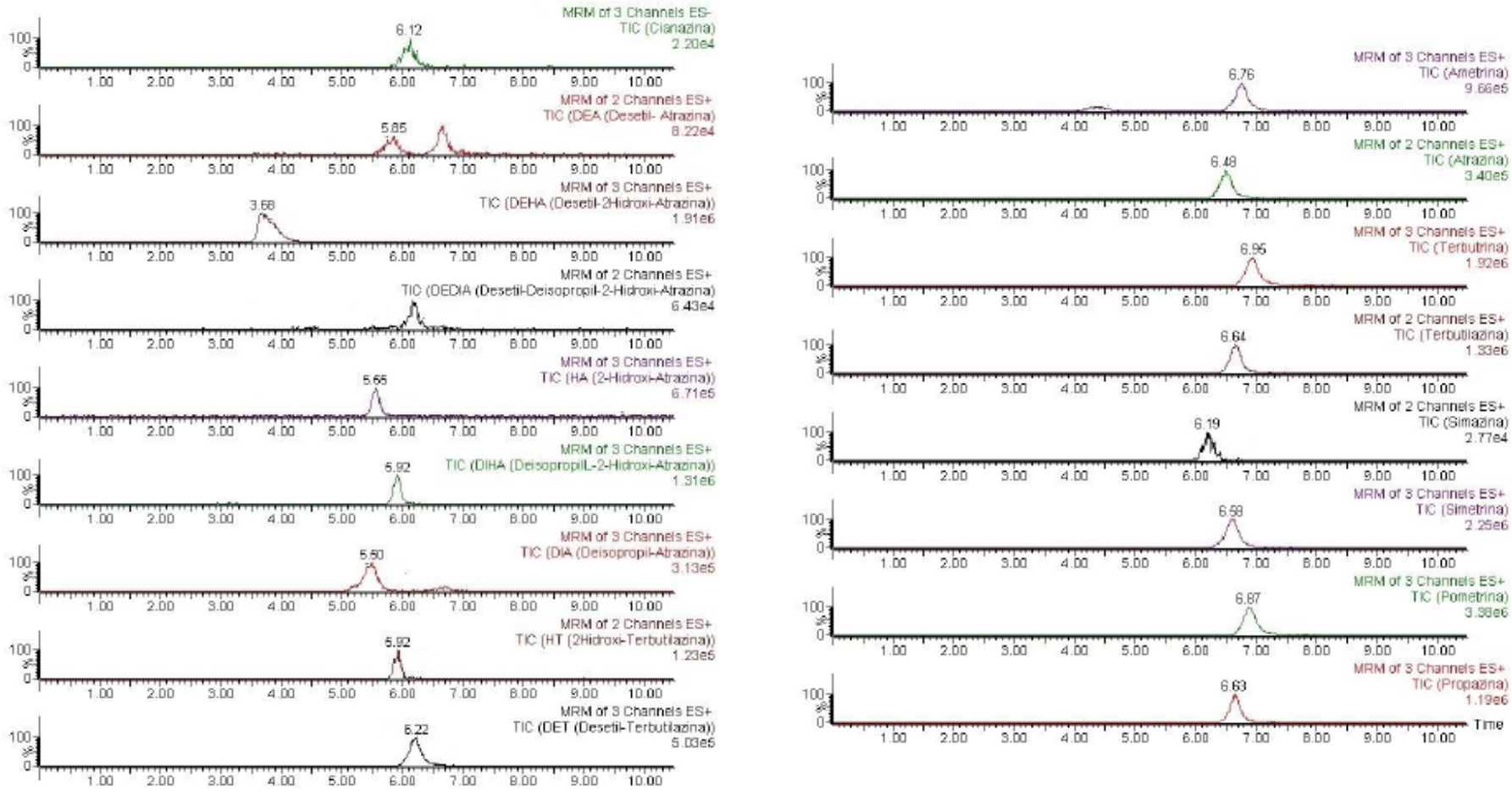

Figure 5. UPLC-MS/MS chromatograms of herbicides and degradation products from a seawater sample spiked with 0.1 µg L^{-1} for the nine triazines, DEA, DET and HA and 1 µg L^{-1} for DEDIA, DEHA, DIA, DIHA and HT

Table 1. Retention time and MS/MS optimized parameters for the studied pesticides

Compound	Rt (min)	Precursor ion (m/z)	Product ion (m/z)	Cone (V)	Collision energy (eV)
DEHA	3.68	170,1	43,1	36	34
			86,0		22
			128,0		16
DIA	5.50	174,0	68,0	36	26
			96,0		16
			104,0		26
HA	5.55	198,1	69,0	42	36
			86,0		22
			114,0		22
DEA	5.85	188,0	79,0	50	16
			146,1		12
HT	5.92	332,1	234,1	12	14
			256,1		6
DIHA	5.92	156,1	69,0	32	24
			86,0		18
			114,0		14
Cyanazine	6.12	239,1	81,0	20	16
			176,1		14
			212,0		10
Simazine	6.19	202,5	104,0	38	28
			146,0		18
DET	6.22	202,1	79,0	34	22
			104,0		28
			146,0		14
DEDIA	6.24	146,1	43,0	38	24
			79,0		18
Atrazine	6.48	216,1	96,1	34	24
			174,1		20
Simetryn	6.58	214,1	68,0	42	32
			96,1		26
			124,1		20
Propazine	6.63	230,1	79,0	40	30
			104,0		34
			146,0		24
Terbuthylazine	6.64	230,1	146,0	36	22
			174,0		16
Ametryn	6.76	228,1	68,1	42	38
			91,0		26
			96,1		26
Prometryn	6.87	242,1	43,1	44	36
			68,1		34
			158,0		24
Terbutryn	6.95	242,1	91,0	34	26
			96,1		30
			186,1		18

Table 2. Validation of the on-line SPE-UPLC-MS/MS method

Compound	Concentration range (µg L^{-1})	LOD (µg L^{-1})	LOQ (µg L^{-1})	Determination coefficient (R^2)	[a] Intercept interval	[a] Slope interval	[a] CV slope (%)	Intraday precision RSD (%)[b]		Inter-day precision RSD (%)[c]	Recovery (%)
								Level 1	Level 2		
Ametryn	0.025-0.5	0.017	0.051	0.9992	14051.3 ± 0.0	853516 ± 0	$3.7\ 10^{-6}$	0.6	0.1	0.1	86.8 ± 0.6
Atrazine	0.025-0.5	0.019	0.057	0.9990	6409.8 ± 0.0	351161 ± 0	$1.0\ 10^{-5}$	1.8	0.4	0.4	81.0 ± 1.8
Cyanazine	0.025-0.5	0.018	0.054	0.9991	125.9 ± 0.0	32941 ± 0	$1.0\ 10^{-4}$	1.9	0.5	0.8	90.0 ± 1.7
DEA	0.025-0.5	0.016	0.048	0.9993	1615.0 ± 0.0	118441 ± 0	$2.5\ 10^{-5}$	2.2	0.6	2.1	88.9 ± 0.6
DEDIA	0.25-5	0.152	0.459	0.9995	138.5 ± 0.1	6398 ± 0	$5.0\ 10^{-4}$	0.8	0.5	0.2	85.7 ± 2.3
DEHA	0.25-5	0.133	0.402	0.9995	3471.7 ± 0.1	63402 ± 0	$4.0\ 10^{-5}$	2.5	0.6	1.2	89.7 ± 0.1
DET	0.025-0.5	0.018	0.056	0.9991	16205.8 ± 0.0	582235 ± 0	$6.0\ 10^{-6}$	0.7	0.3	0.3	90.8 ± 0.2
DIA	0.25-5	0.168	0.508	0.9992	1391.7 ± 0.1	36918 ± 0	$8.7\ 10^{-5}$	1.3	0.3	0.5	92.5 ± 0.3
DIHA	0.25-5	0.217	0.657	0.9987	21062.2 ± 0.1	48449 ± 0	$8.5\ 10^{-5}$	1.1	0.2	0.3	86.5 ± 0.2
HA	0.025-0.5	0.020	0.061	0.9989	5670.2 ± 0.0	456262 ± 0	$8.4\ 10^{-6}$	0.9	0.3	0.6	96.1 ± 0.3
HT	0.25-5	0.179	0.542	0.9991	5853.6 ± 0.1	4430 ± 0	$7.7\ 10^{-4}$	3.9	0.4	0.7	82.4 ± 0.9
Prometryn	0.025-0.5	0.018	0.053	0.9991	43721.4 ± 0.0	4009880 ± 0	$8.4\ 10^{-7}$	0.1	0.0	0.0	91.7 ± 1.0
Propazine	0.025-0.5	0.018	0.054	0.9991	9630.0 ± 0.0	1108789 ± 0	$3.1\ 10^{-6}$	1.1	0.1	0.2	87.7 ± 3.1
Simazine	0.025-0.5	0.019	0.057	0.9990	832.9 ± 0.0	24356 ± 0	$1.5\ 10^{-4}$	1.0	0.4	1.7	83.3 ± 0.1
Simetryn	0.025-0.5	0.010	0.032	0.9997	40018.7 ± 0.0	1674051 ± 0	$1.2\ 10^{-6}$	0.1	0.2	0.2	80.3 ± 0.2
Terbuthylazine	0.025-0.5	0.018	0.055	0.9991	22946.4 ± 0.0	1260632 ± 0	$2.8\ 10^{-6}$	2.7	0.1	0.2	92.2 ± 3.1
Terbutryn	0.025-0.5	0.008	0.023	0.9998	17886.2 ± 0.0	3042096 ± 0	$4.8\ 10^{-7}$	0.4	0.0	0.1	99.8 ± 0.2

[a] Confidence level 95%

[b] 0.025 and 0.1 µg L^{-1} for the nine triazines, DEA, DET and HA and 0.25 and 1 µg L^{-1} for DEDIA, DEHA, DIA, DIHA and HT

[c] 0.1 µg L^{-1} for the nine triazines, DEA, DET and HA and 1 µg L^{-1} for DEDIA, DEHA, DIA, DIHA and HT

ON-LINE SOLID-PHASE EXTRACTION METHOD FOR DETERMINATION OF TRIAZINE HERBICIDES AND DEGRADATION PRODUCTS IN SEAWATER BY ULTRA-PRESSURE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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INTRODUCTION

The Directive 2013/39/EU calls the attention on the important role of monitoring emerging pollutants that are not regularly considered in monitoring programs but can have toxicological effects [1]. This Directive includes three triazines (atrazine, simazine and terbutryn). The impact due to herbicides tends to be underestimated when only the triazines are analysed. Therefore, the main degradation products should be included in current analytical methods to obtain a better knowledge of water quality regarding herbicides contamination [2]. Furthermore, the monitoring of these compounds in marine ecosystems situated close to areas of intensive horticulture is of great interest. For this reason, a fast, simple, selective and sensitive method based on on-line solid-phase extraction followed by UPLC-MS/MS was developed for the quantification of 17 compounds (nine triazine herbicides as well as their main degradation products) in seawater. The proposed method was applied to determine the target herbicides in seawater samples from beaches of Matosinhos (Portugal).

EXPERIMENTAL METHOD

UPLC-MS/MS Determination

Acquity UPLC Waters with an UPLC C18 reverse-phase bounded to ethylene-bridged hybrid (BEH) column (2.1 mm x 100 mm, 1.7 µm) with a C18 BEH pre-column (2.1 mm x 5 mm, 1.7 µm)

Acquity triple-quadrupole mass spectrometer Waters with an ESI source. MRM conditions used for the analysis of pesticides are shown in **Table 1**

Gradient elution

Mobile phase A: 5 mM CH_3COONH_4 in H_2O
Mobile phase B: 5 mM CH_3COONH_4 in MeOH

Initially 95% (A) decreased to 0% in 5 min and holding in it for 3 min and returned to initial conditions in 0.1 min

Sample volume: 5 mL

Chromatographic run: 11 min

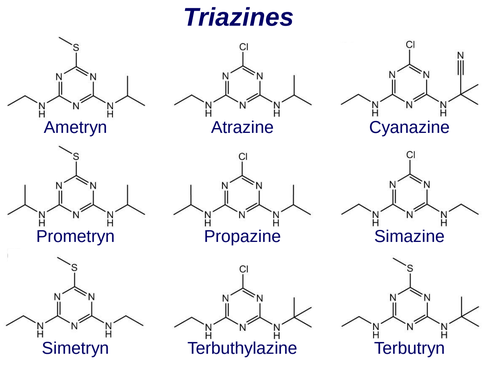
Sample site location

Samples were collected from ten beaches nearby a zone of intensive horticulture

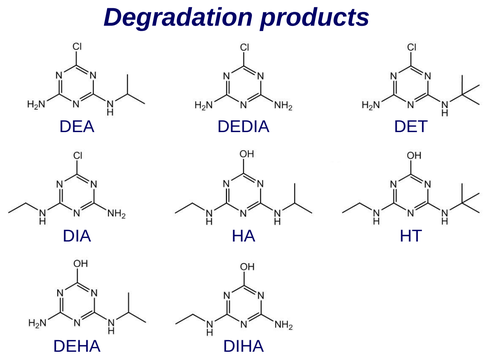
Upon reception samples were filtered (PTFE syringe filter)

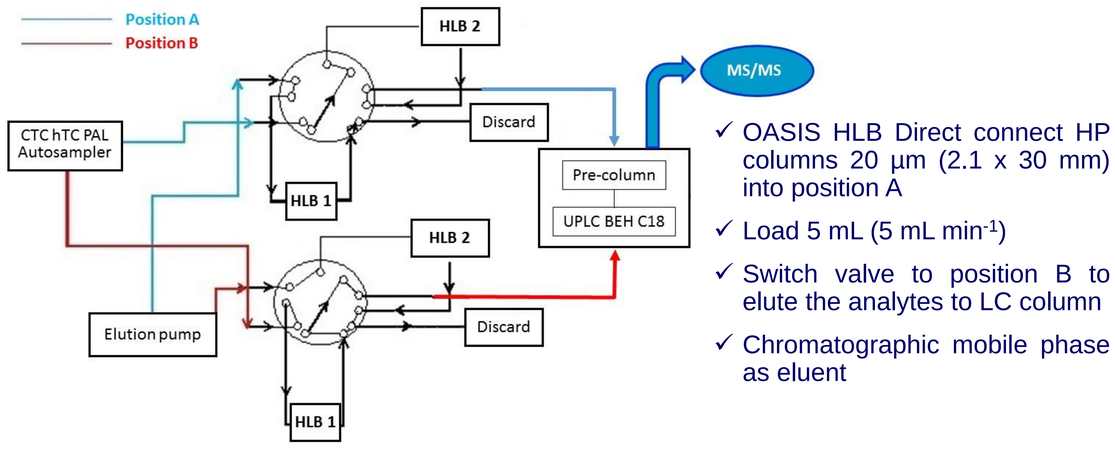
Matosinhos (Portugal)

Triazines



Degradation products





- ✓ OASIS HLB Direct connect HP columns 20 µm (2.1 x 30 mm) into position A
- ✓ Load 5 mL (5 mL min^{-1})
- ✓ Switch valve to position B to elute the analytes to LC column
- ✓ Chromatographic mobile phase as eluent

Scheme of the on-line SPE-UPLC-MS/MS

RESULTS AND DISCUSSION

Optimization of UPLC-MS/MS conditions

MS/MS parameters

Optimization was performed by infusion of individual standard solutions. (see **Table 1**)

Cyanazine was analysed in negative mode and remaining compounds in positive mode.

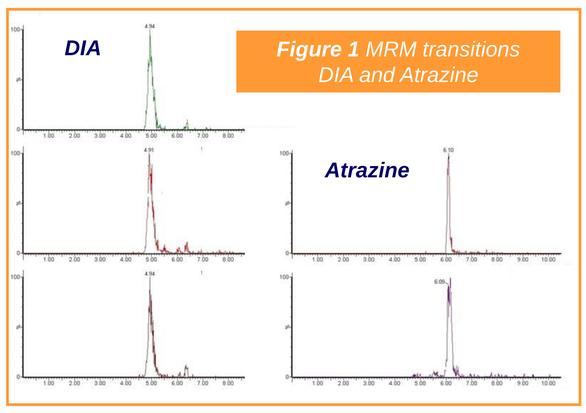


Figure 1 *MRM transitions DIA and Atrazine*

UPLC conditions

Mobile phases composition was evaluated
5 mM CH_3COONH_4 in both mobile phases ✓

Various gradients were also tested
Conditions described in Experimental section ✓

Table 1: Retention time and MS/MS

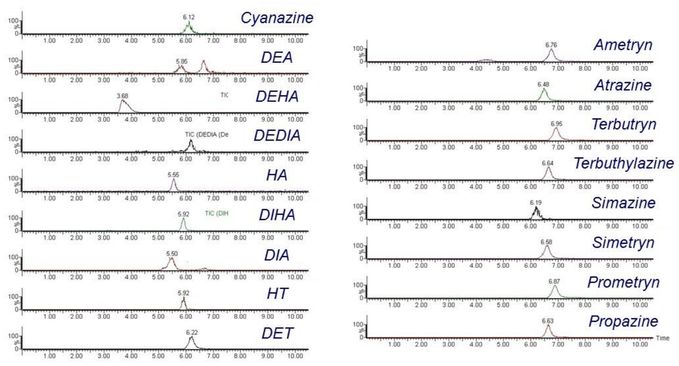
Compound	Rt (min)	Precursor ion (m/z)	Product ion (m/z)	Cone (V)	Collision energy (eV)
DEHA	3.68	170,1	43	36	34
			86		22
			128		16
DIA	5.50	174,0	68	36	26
			96		16
			104		26
HA	5.55	198,1	69	42	36
			86		22
			114		22
DEA	5.85	188,0	79	50	16
			146		12
HT	5.92	332,1	234	12	14
			256		6
DIHA	5.92	156,1	69	32	24
			86		18
			114		14
Cyanazine	6.12	239,1	81	20	16
			176		14
			212		10
Simazine	6.19	202,5	104	38	28
			146		18
DET	6.22	202,1	79	34	22
			104		28
			146		14
DEDIA	6.24	146,1	43	38	24
			79		18
Atrazine	6.48	216,1	96	34	24
			174		20
Simetryn	6.58	214,1	68	42	32
			96		26
			124		20
Propazine	6.63	230,1	79	40	30
			104		34
			146		24
Terbuthylazine	6.64	230,1	146	36	22
			174		16
Ametryn	6.76	228,1	68	42	38
			91		26
			96		26
Prometryn	6.87	242,1	43	44	36
			68		34
			158		24
Terbutryn	6.95	242,1	91	34	26
			96		30
			186		18

Method performance

The method was validated by estimation of the linearity, LODs and LOQs, recovery and precision. Results are presented in **Table 2**

Compound	Concentration range (µg L^{-1})	LOD (µg L^{-1})	LOQ (µg L^{-1})	Determination Coefficient (R^2)	Intra-day precision RSD(%)[a] Level 1	Intra-day precision RSD(%)[a] Level 2	Inter-day precision RSD (%)[b]	Recovery (%)[b]
Ametryn	0.025-0.5	0.017	0.051	0.9992	0.6	0.1	0.1	86.8 ± 0.6
Atrazine	0.025-0.5	0.019	0.057	0.9990	1.8	0.4	0.4	81.0 ± 1.8
Cyanazine	0.025-0.5	0.018	0.054	0.9991	1.9	0.5	0.8	90.0 ± 1.7
DEA	0.025-0.5	0.016	0.048	0.9993	2.2	0.6	2.1	88.9 ± 0.6
DEDIA	0.25-5	0.152	0.459	0.9995	0.8	0.5	0.2	85.7 ± 2.3
DEHA	0.25-5	0.133	0.402	0.9995	2.5	0.6	1.2	89.7 ± 0.1
DET	0.025-0.5	0.018	0.056	0.9991	0.7	0.3	0.3	90.8 ± 0.2
DIA	0.25-5	0.168	0.508	0.9992	1.3	0.3	0.5	92.5 ± 0.3
DIHA	0.25-5	0.217	0.657	0.9987	1.1	0.2	0.3	86.5 ± 0.2
HA	0.025-0.5	0.020	0.061	0.9989	0.9	0.3	0.6	96.1 ± 0.3
HT	0.25-5	0.179	0.542	0.9991	3.9	0.4	0.7	82.4 ± 0.9
Prometryn	0.025-0.5	0.018	0.053	0.9991	0.1	0.0	0.0	91.7 ± 1.0
Propazine	0.025-0.5	0.018	0.054	0.9991	1.1	0.1	0.2	87.7 ± 3.1
Simazine	0.025-0.5	0.019	0.057	0.9990	1.0	0.4	1.7	83.3 ± 0.1
Simetryn	0.025-0.5	0.010	0.032	0.9997	0.1	0.2	0.2	80.3 ± 0.2
Terbuthylazine	0.025-0.5	0.018	0.055	0.9991	2.7	0.1	0.2	92.2 ± 3.1
Terbutryn	0.025-0.5	0.008	0.023	0.9998	0.4	0.0	0.1	99.8 ± 0.2

Figure 2
Chromatograms from spiked seawater sample: 0.1 µg L^{-1} (nine triazines, DEA, DET and HA) and 1 µg L^{-1} (DEDIA, DEHA, DIA, DIHA and HT)



a 0.025 and 0.1 µg L^{-1} DEA, DET, HA and triazines 0.25 and 1 µg L^{-1} DIA, DEDIA, DEHA, DIHA and HT
b 0.1 µg L^{-1} DEA, DET, HA and triazines; 1 µg L^{-1} DIA, DEDIA, DEHA, DIHA and HT

Application

The method was applied to seawater samples. Although, none of the herbicides were detected in the 10 seawater samples from the seashore of Matosinhos, the monitoring of their levels in marine ecosystems situated close to areas of intensive horticulture is of great local interest both economic and environmental.

CONCLUSIONS

- On-line SPE-UPLC-MS/MS has shown to be a fast, sensitive and robust alternative to traditional off-line SPE for the analysis of triazines and their main degradation products in seawater samples.
- The method is suitable to be used in routine analysis due to sample pre-treatment is not required. Furthermore, it reduces chemical wastes because of the use of a minimal amount of extraction solvents.
- The method has shown suitable precision and good recovery for all compounds and the LOQs enable the determination of these pollutants at the levels required by EU legislation [1] using only 5 mL of sample.
- Although a few methods based on SPE combined with UPLC-MS/MS have been used to measure triazines and degradation products in river waters, there are not studies in seawater. Furthermore, an important difference of the method with previously described methodology is the determination of a greater number of degradation products simultaneously with triazines.

Acknowledgments

This work was supported by Ministerio de Economía y Competitividad (CTM2013-48194-C3-2-R) and Program of Consolidation and Structuring of Units of Competitive Investigation of the University System of Galicia (Xunta de Galicia) potentially cofounded by European Regional Development Fund (ERDF) in the frame of the operative Program of Galicia 2007-2013 (reference: GRC2013-047). N. Rodríguez-González thanks the "Xunta de Galicia" and Campus do Mar (International Campus of Excellence) for the concession of her PhD. Grant and the Universidade da Coruña for the concession of her INDITEX-UDC 2015 Grant for her stay in Porto.

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CAPÍTULO III

SEDIMENTO

INTRODUCCIÓN

En este capítulo, se optimizó un método para la extracción de las nueve triazinas en sedimentos empleando dispersión de la matriz en fase sólida. Una vez optimizado el método de extracción, se realizó la adaptación del mismo para la extracción de los productos de degradación. Además, para completar el estudio, el método de SPE propuesto en el capítulo II para la extracción de triazinas en agua de mar, se modificó para poder llevar a cabo la extracción de todos los compuestos de interés. Para cada uno de estos métodos se realizó tanto la optimización de las distintas etapas, como la validación y su aplicación a muestras de una zona de la ría de A Coruña.

En primer lugar, se desarrolló un método eficaz y simple basado en la técnica de extracción mediante dispersión de la matriz en fase sólida para la determinación simultánea de nueve triazinas (Ametrina, Atrazina, Cianazina, Prometrina, Propazina, Simazina, Simetrina, Terbutilazina y Terbutrina) en muestras de sedimento. Se probaron distintos tipos de adsorbentes (C_{18}, tierra de diatomeas y ENVI-Carb), diferentes coadsorbentes (SAX/PSA, ENVI-Carb II, ENVI-Florisil y ENVI-carb II/PSA) y los disolventes que se ensayaron en la elución fueron acetato de etilo y acetonitrilo. Después de seleccionar el agente dispersante, los coadsorbentes y los eluyentes, se procedió a la validación del método. La determinación de las triazinas se llevó a cabo mediante cromatografía de líquidos y detección con red de diodos (HPLC-DAD) y se realizó su confirmación mediante cromatografía líquida-espectrometría de masas (LC-ESI-MS/MS). Finalmente, el método validado se aplicó al análisis de las nueve triazinas en muestras reales de sedimento marino de la ría de A Coruña.

Debido a la importancia que tiene analizar no sólo las triazinas sino también sus principales productos de degradación en los diferentes compartimentos ambientales, se realizó un estudio para la determinación de las triazinas y sus principales productos de degradación en dos matrices del medio marino (agua de mar y sedimentos), empleando en ambos casos el mismo equipo para su determinación (LC-ESI-MS/MS). En las muestras de sedimentos, el método de extracción empleado ha sido MSPD y en el caso del agua de mar, SPE. Se llevó a cabo la optimización de los procesos de extracción, separación, identificación y cuantificación de las nueve triazinas y sus principales productos de degradación (DEA, DEDIA, DEHA, DET, DIA, DIHA, HA y HT), así como la validación de los métodos. Por último, los métodos validados se han aplicado a la determinación de los compuestos estudiados en muestras de agua de mar y sedimentos marinos de varios puntos de la ría de A Coruña.

Publicaciones:

Development of a matrix solid phase dispersion methodology for the determination of triazine herbicides in marine sediments. Rodríguez-González, N., González-Castro, M.J., Beceiro-González, E., Muniategui-Lorenzo, S. **Artículo enviado para su publicación.**

Determination of triazine herbicides and their degradation products in seawater and marine sediments using liquid chromatography-tandem mass spectrometry. (2016). Rodríguez-González, N., Uzal-Varela, R., González-Castro, M.J., Muniategui-Lorenzo, S., Beceiro-González, E. **Artículo aceptado en *Environmental Science and Pollution Research.***

Congresos:

Development of a matrix solid phase dispersion methodology for the determination of triazine herbicides in marine sediments. (2016). Rodríguez-González, N., González-Castro, M.J., Beceiro-González, E., Muniategui-Lorenzo, S. *"9th European Conference on Pesticides and Related Organic Micropollutants in the Environment"* and *"15th Symposium on Chemistry and Fate of Modern Pesticides"*. Páginas 167-168. Santiago de Compostela (ISBN: 978-84-945958-1-3). **Anexo II-III, página 365.**

DEVELOPMENT OF A MATRIX SOLID PHASE METHODOLOGY FOR THE DETERMINATION OF TRIAZINE HERBICIDES IN MARINE SEDIMENTS

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ABSTRACT

A method based on Matrix Solid Phase Dispersion (MSPD) for the determination of nine triazines in marine sediments was optimized in terms of dispersant agents, co-sorbents and elution solvents. Three dispersing agents: octadecylsilyl bonded silica (C18), graphitized carbon black (GCB) and diatomaceous earth, and four clean-up co-sorbents: Florisil, GCB, primary and secondary amine (PSA)/strong anion exchanger (SAX), and GCB/PSA were assayed. The best results were obtained with GCB as dispersing agent, without co-sorbent, using 20 mL of ethyl acetate as elution solvent. Finally the extract was concentrated to dryness, re-constituted with 1 mL methanol and determined by HPLC-DAD. The analytical recoveries obtained were close to 100 % and repeatability and reproducibility were below than 3.5 % for all compounds. The linearity of the calibration curves was excellent in matrix matched standards, and yielded the coefficients of determination $\geq$ 0.9992 for all the target analytes. The LOQ values ranged from 0.022 to 0.037 mg kg^{-1}. Finally the method was applied to analyse the target compounds in marine sediment samples from the estuary of A Coruña (Galicia, NW of Spain).

Keywords: Diode array detection. High performance liquid chromatography. Matrix solid phase dispersion. Sediment. Triazines.

1. Introduction

Triazines are a group of herbicides that are present in the ten most-used herbicide formulations in Europe. Triazines have been used extensively as herbicides to provide pre- and post-emergence of grasses, crops and many weeds in cereals [1] but they are also employed for non-agricultural purposes including soil sterilization and road maintenance [2]. However, it has been estimated that after their application, a large proportion remains in the environment [3] and because of their widespread, persistence and mobility fluxes of these compounds reach the marine environment.

The contamination of the marine environment from herbicides is of great concern because of their high distribution in the aquatic system and of their toxic properties. Therefore, triazine herbicides are considered as an important class of chemical pollutants and they are also in the list of chemical pollutants that need to be more monitored. Atrazine and simazine have been included in the Endocrine Disruption Screening Program by the U.S. Environmental Protection Agency [4]. On the other hand, the European Union has also included simazine and atrazine in the list of 33 priority substances in the EU Water Framework Directive (2000/60/EC) [5] by way of Decision 2455/2001/EC [6]. Moreover, the Directive 2008/105/EC [7] sets the Environmental Quality Standards (EQS) for these compounds in water and also committees the Member States to set EQS for these compounds in sediments and/or biota at national level. Furthermore, the European Directive requires the Member States to monitor sediment at an adequate frequency to provide sufficient data of those priority substances that tend to accumulate in sediment. Therefore it is necessary to have accurate, effective, simple and fast methods of analysis for these compounds which enable to improve the knowledge and data available on sources of priority substances and ways in which pollution occurs.

Sediments provide habitat for many benthic and epibenthic organisms and are an important component of aquatic system [8]. Sediments are also considered to be compartments of accumulation of contaminants brought by the water column, therefore the analysis of sediments should be included in environmental studies because they are the result of the integration of all processes that occur in an aquatic ecosystem [9]. The sediment properties which influence the movement of pesticides include texture, permeability and organic matter content. Pesticide retention in sediment is mainly attributed to the clay and organic matter of the soil, which provides a number of bonding sites for pesticides; usually, hydrophobic pesticides have a stronger tendency to bind [10]. Consequently, sediments may act as long-term sources of these compounds into the aquatic environment; in fact, because of the sorption capacity of sediment for organic contaminants, sediments have been proposed as adsorbents to reduce the environmental impact of pesticides [2].

Triazine herbicides have been detected and monitored in sediments [10-13], and they have geochemical interest as markers in identifying origin of sedimentary deposits and evolution in aquatic environments [14]. The more frequently used methodologies for the analysis of triazines in sediments employ solvent extraction procedures such as soxhlet [15], mechanical shaken [2,3], sonication [8,16], microwave-assisted extraction (MAE) [10,17] and pressurized liquid extraction (PLE) [12,14]. Nevertheless, they generally need to add a clean-up step to decrease the presence of interferents in the final extract to reduce the detection limits of the methods and to avoid inaccurate results in the

chromatographic determination. In the last years, different innovation procedures have been developed and applied for the determination of pollutants in complex matrices with improved capabilities, reduced clean up and concentration steps, the avoidance of toxic solvents and improved limits of detection. In this context, extraction techniques such as QuEChERS (standing for Quick, Easy, Cheap, Effective, Rugged and Safe) [9,13] and matrix solid-phase dispersion (MSPD) [18] appear to be appropriate for the determination of triazines in sediments.

MSPD is an extraction procedure which combines aspects of several analytical techniques allowing sample homogenization, disruption, extraction, fractionation and clean-up within a single process [19]. In MSDP the solid sample is blended in a mortar with a sorbent to obtain complete disruption and dispersion of the sample. Then the mixture is packed into a column and analytes are eluted with an appropriate solvent. Often, a co-sorbent material is placed at the bottom of the column to be filled with the blended sample to assist the extract clean-up. The key factors for the success of MSPD are its feasibility, flexibility, versatility, high throughput, low cost and rapidity. MSPD methods have been developed for extraction of pesticides and other contaminants from both vegetable and animal matrices; however application of MSPD to environmental solid samples, such as soil or sediments, are relatively few compared to solvent extraction techniques [20]. As far as triazines is concern, to the best of our knowledge, there are only two references in the literature devoted to the determination of triazines in soil and sediments by MSPD, and furthermore few triazines are included in these studies [18,21].

Therefore the aim of this work was the development and validation of a method based on Matrix Solid Phase Dispersion for the simultaneous determination of nine triazine herbicides in marine sediments. As far as we know, no studies using MSPD have been done to extract these chemicals residues from marine sediments. The developed method was used for the determination of target compounds in marine sediment samples from ten points susceptible to contamination by triazines from estuary of A Coruña (NW Spain).

2. Material and methods

2.1. Sample collection

Marine sediment samples were collected at 10 potential polluted sites by triazines from estuary of A Coruña (NW Spain). The estuary is formed by the mouth of Mero River and it is also fed by many small rivers and brooks that traverse areas dedicated to agriculture. The sampling locations were selected by their proximity to one or more potential sources of contamination by triazine herbicides. Figure 1 shows a map of sampling points, where the target compounds were also analysed in sea water samples

[22]. Additionally, uncontaminated marine sediments collected from the Ares estuary (A Coruña, NW Spain) were used for optimization and validation of MSPD procedure.

After collection, samples were homogenized, frozen at −20 °C and freeze-dried. Then they were pulverized by using a vibrating ball mill and sieved through a 0.5 mm mesh. Finally samples were stored in amber glass bottles with hermetic seals out of light exposure until analysis.

2.2. Chemicals

(a) *Herbicide standards*- Herbicides (ametryn, atrazine, cyanazine, prometryn, propazine, simazine, simetryn, terbuthylazine and terbutryn) analytical standards were supplied by Sigma-Aldrich (Inc. St. Louis, MO, USA). The individual stock standard solutions of 1000 mg L^{-1} were prepared in methanol by exact weighing of high-purity substances and stored at -18º C in the dark. Then a mixture of all the compounds was prepared in methanol containing 10 mg L^{-1} each individual triazine and stored at -18º C. All working solutions were daily prepared by appropriate dilution of the 10 mg L^{-1} standard solutions with methanol.

(b) *Solvents* –Acetonitrile (HPLC grade) and ethyl acetate (PAR, solvents for analysis of pesticide residues by GC) for instrumental analysis were from Panreac (Barcelona, Spain). Methanol was superpurity Solvents from Romil (Cambridge, UK). Milli-Q water was obtained from a purification system from Millipore (Billerica, MA).

(c) *Sorbents – SPE Bulk packing:* ENVI-18, diatomaceous earth and ENVI-Carb, and *SPE tubes*: ENVI-Florisil (1 g), ENVI-Carb (1 g), ENVI-Carb-II/PSA (500 mg/500 mg), SAX/PSA (500 mg/500 mg) were from Sigma-Aldrich (Inc. St Louis, MO, USA).

2.3. Materials and apparatus

Empty glass solid phase extraction syringes (6 mL capacity) and 20 µm polyethylene frits were purchased from Sigma-Aldrich (Inc. St Louis, MO, USA). Syringe polytetrafluoroethylene (PTFE) filters of 0.45 µm were obtained from Teknocroma (Barcelona, Spain).

A Visiprep® vacuum distribution manifold from Supelco (Bellefonte, PA, USA) was employed in the purification step. A Büchi R-3000 rotary evaporator (Büchi Labortechnic AG, Flawil, Switzerland) was used in the evaporation step.

Chromatographic analyses were carried out in a high performance liquid chromatography-diode array detector (HPLC-DAD). The system consisted of a 2695 pump with a 996 Diode Array Detector from Waters (Milford, MA, USA). The column was a stainless steel column (150 mm x 4.6 mm ID, particle size 5 µm) packed with Hypersil GOLD C_{18} chemical bonded phase from Thermo Scientific (Austin, TX, USA).

2.4. Extraction procedure

Optimization experiments of the several sorbents tested were carried out by analysing a triazine-free freeze-dried sediment sample spiked with the triazines to obtain a final concentration of each analyte in the sediment of 1 mg kg^{-1}. Under final working conditions, 1.0000 g of freeze-dried sediment sample was homogenised with 1 g of ENVI-Carb in a glass mortar with a pestle for 5 min. The final mixture was transferred into a 6 mL glass syringe and once packed, MSPD columns were connected to a Visiprep® vacuum distribution manifold. Elution was performed with 20 mL of ethyl acetate and the obtained eluate was evaporated to a drop in rotary-evaporator and got to dryness by a gentle nitrogen stream. The residue was reconstituted in 1 mL methanol and the solution was filtered through of a 0.45 µm syringe filter of PTFE. As an illustration, In Figure 2, MSPD procedure.

2.5. HPLC-DAD conditions

The chromatographic analysis was carried out using the following ACN:H_2O gradient elution: ACN initial percentage of 30 % (8 min), increased linearly to 40 % in 5 min; increased to 50 % in 5 min, after which the percentage was returned to the initial conditions in 9 min. A constant mobile phase flow rate of 1 mL min^{-1} and 20 µL of sample volume were used.

The absorbance was measured continuously in the 200-400 nm range and peaks areas quantification were carried out at 222.7 nm in order to achieve maximum sensitivity. All triazine herbicides were identified initially by retention time and then by spectral identification contrasting the spectrum with a standard library created in the same wavelength interval.

3. Results and discussion

3.1. MSPD optimization

The effectiveness of an MSPD procedure depends on the dispersant sorbent/solvent combination. In addition to this parameters, the performance of MSPD protocols may be affected by the characteristics of the clean-up co-sorbent. Most MSPD procedures reported for the extraction of contaminants in environmental samples use RP-C18 [23,24] and diatomaceous earth [25,26] as dispersants, usually followed by a clean-up step using a normal phase co-sorbent.

For optimization of the MSPD procedure, the following different experimental factors were taking into consideration: type of dispersing agent and sorbents used for the clean-up, and elution solvent. Extraction conditions were evaluated by spiking 1.000 g of pesticide free sediment sample at the 1 mg kg^{-1} level. For preliminary experiments RP-C18 was chosen as dispersant, because it is most popular than diatomaceous earth for

the extraction of these compounds from plant and animal tissues [27,28]; furthermore, the only reference devoted to determination of triazines in sediment employs C18 as dispersing agent [18]. For this purpose, ENVI-18 (1 g) as dispersant was tested with four online clean-up co-sorbents: ENVI-Florisil (1 g), ENVI-Carb (1 g), ENVI-Carb-II/PSA (500 mg/500 mg) and SAX/PSA (500 mg/500 mg). Based on our experience on the MSPD technique in the analysis of the target compounds in biota [29,30], employing reversed phase materials as dispersants and a clean-up co-sorbent, elution of triazines was performed with 20 mL of ethyl acetate and 5 mL of acetonitrile. All assays provided extracts clean enough to be injected, however experiments carried out using SAX/PSA and ENVI-Florisil led to yellow extracts, whereas ENVI- Carb and ENVI-Carb-II/PSA provided colourless eluates. As it can be seen in Figure 3, satisfactory recoveries (>80%) for all triazines were achieved with the four systems assayed.

Because of experiments employing co-sorbents were clean enough to be injected, in order to reduce costs, next assays were carried out to check the needless of an additional co-sorbent. For this purpose, on the basis of preliminary results, three dispersants without using clean-up co-sorbent were evaluated: ENVI-18, diatomaceous earth and ENVI-Carb. Elution of the triazines was also carried performed with 20 mL of ethyl acetate and 5 mL of acetonitrile All MSPD extracts were clean enough to be directly subjected to instrumental analysis. The efficiency of the three dispersants in terms of recoveries (Figure 4) was satisfactory for all triazines in all cases (>85%). On the other hand, experiments carried out using ENVI-18 and diatomaceous earth led to yellow extracts, whereas ENVI-Carb was the only one that gave colourless eluates. Thus ENVI-Carb as dispersant, without an on-line clean-up sorbent, was selected for the extraction of these compounds from marine sediments.

Thereafter, due to the use of a clean-up co-sorbent was not necessary, the elution profile of target analytes was investigated collecting five eluates of 5 mL of the above solvent in separated vessels. The obtained results showed that at least 10 mL of ethyl acetate were necessary for recovering all compounds from the MSPD system except for symetrin, which required an additional volume of 10 mL of ethyl acetate. The last fraction, corresponding to acetonitrile, contained non-detectable amount of the target analytes. Thus, 1 g of ENVI- Carb as dispersant and 20 mL of ethyl acetate as elution solvent were selected. Figure 5 shows the chromatograms corresponding to unspiked and spiked sediment sample analysed under the considered procedure.

3.2. Method validation

For quantification purposes matrix matched standards prepared by spiking the final extracts from blank samples of sediment with different levels of triazines were prepared. The linearity of the calibration curves was calculated at a concentration range between

0.1-2 mg kg^{-1} dried sample by duplicate analysis at six different concentration levels. As can be seen in Table 1, excellent linearities were obtained with coefficients of determination (R^2) higher than 0.9992 for all triazines. The limits of detection (LODs) and quantitation (LOQs), were calculated as the minimum amount of target analyte that led to a chromatogram peak with a signal-to-noise ratio of 3 and 10 respectively, with reference to the background noise obtained from blank sediment sample. As shown in Table 1, LODs varied from 0.007 to 0.011 mg kg^{-1} and LOQs from 0.022 to 0.037 mg kg^{-1}. By comparing the LODs obtained by the proposed methodology with those reported using MSPD in soils employing DAD detection [21], better sensitivity is attained.

Precision and trueness of the proposed method were determined by spiking 1.0000 g of uncontaminated marine sediment sample at two concentration levels (0.1 and 1 $mgkg^{-1}$). The trueness of the method was evaluated regarding the recovery assay by analysis of five replicates at each fortification level; the analytical recoveries were close to 100% for both levels (Table 2). The precision of the method, expressed as the relative standard deviation (RSD), was evaluated measuring five replicates samples at the same day (intraday precision) and six replicate samples on three consecutive days (inter-day precision). The RSD values were lower than 3.4 % (Table 2).

The obtained results are in accordance with the Commission Decision 2002/657/EC [31], which establishes "criteria concerning the performance of analytical methods and the interpretation of the results". It is worth noting that the obtained recoveries and relative standard deviation are comparable or even better to those provided by other authors for the determination of some of these pollutants in sediment samples using solvent extraction techniques followed by several clean-up strategies [2,11,14,17], and by MSPD in soils [21] and sediments [18].

3.3. Analysis of estuarine sediment samples

Once the method was validated, it was applied to investigate the presence of the target species in ten marine sediments from the estuary of A Coruña (Galicia, NW of Spain). Although none of them contained detectable amount of target pesticides, studies regarding the presence of these compounds are required on sediments collected from estuaries because these areas are subject to shore side contaminant discharges.

4. Conclusions

The suitability of a procedure based on MSPD for the extraction of nine triazines from sediment samples has been demonstrated for the first time. The method uses graphitized carbon black as dispersant with 20 mL ethyl acetate as elution solvent. The developed method provides satisfactory trueness and precision for the determination of triazines in

marine sediments. The main advantages of this methodology when compared with classical methods of sample preparation to determine these compounds are easy of work-up, fast, low cost, avoidance of clean-up procedure, as well as the significant reduction of organic solvents and energy requirements in agreement with the principles of the Green Chemistry.

The proposed method was applied to the analysis of marine sediments from the estuary of A Coruña (Galicia, NW of Spain). Although the triazines under study were not detected in the samples, the analysis of these compounds in marine sediments is of great interest in order to control the quality of the marine environment.

Acknowledgements

This work has been financially supported by Xunta de Galicia potentially cofunded by European Regional Development Fund (EDRF) (GRC-2013-047) and by the Spanish Ministry of Economy and Competitiveness cofunded by ERDF (CTM2013-48194-C3-2-R). N. Rodríguez-González also thanks the "Xunta de Galicia" for the concession of her Ph.D grant under the program of the International Campus of Excellence "Campus del Mar".

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Figure 1. Location of sampling sites in estuary of A Coruña (Galicia, NW of Spain)

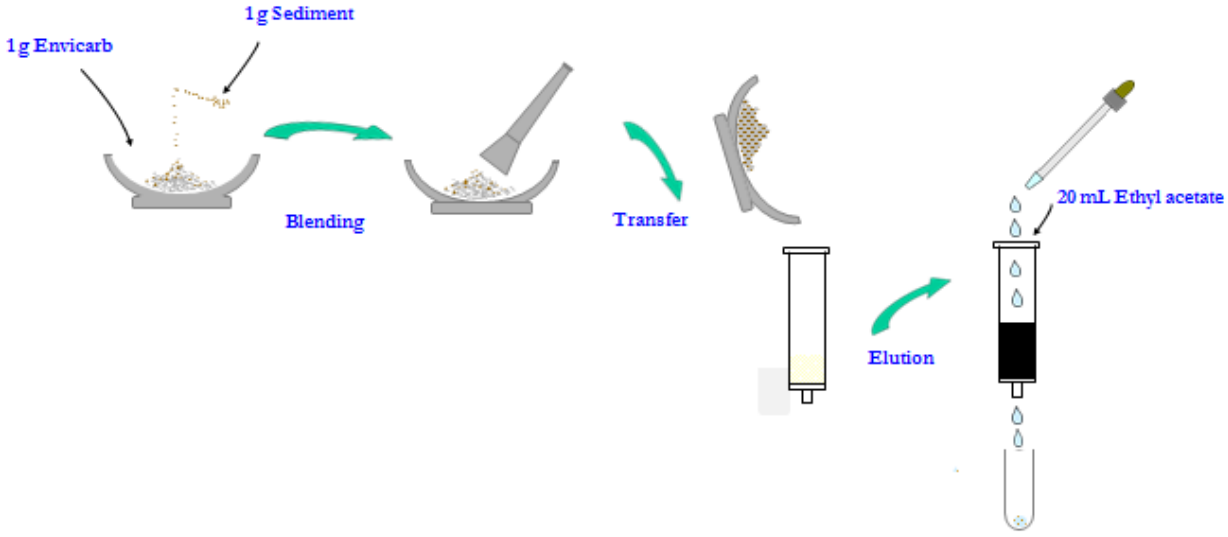


Figure 2. Scheme showing MSPD procedure

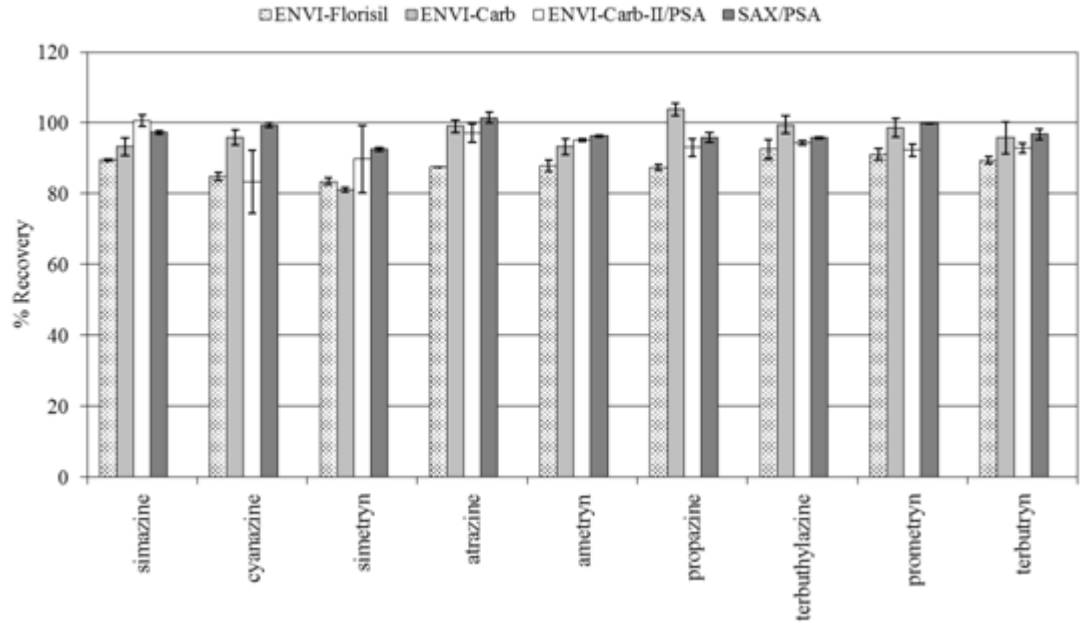


Figure 3. Comparison of analytical recoveries obtained using ENVI-18 as dispersing agent with different clean-up co-sorbents

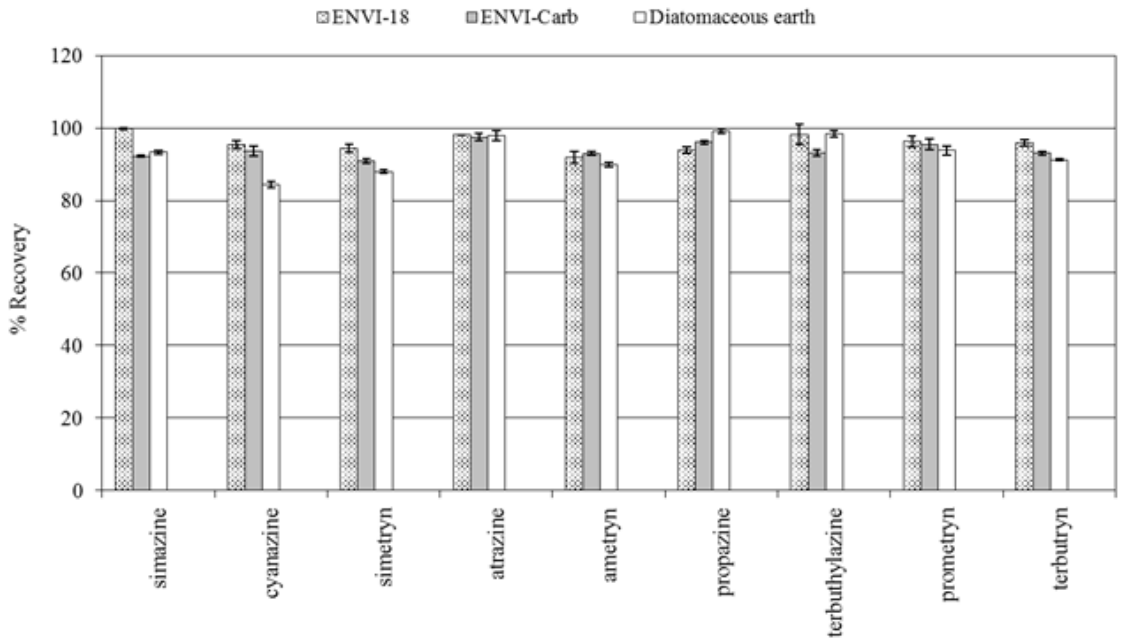
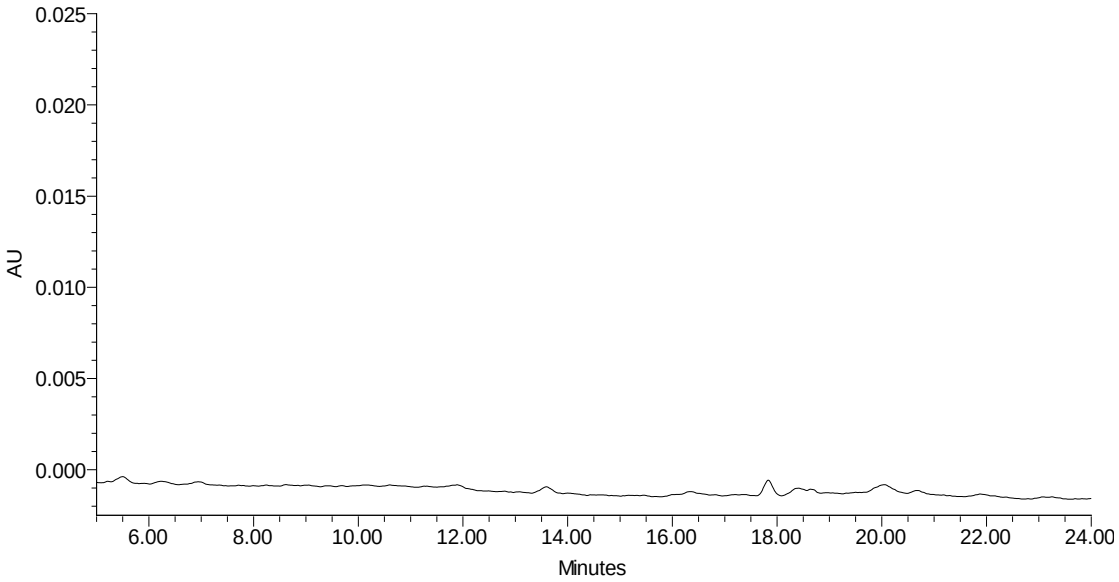


Figure 4. Effect of the type of dispersant sorbent on the extraction efficiency of MSPD

(a)



(b)

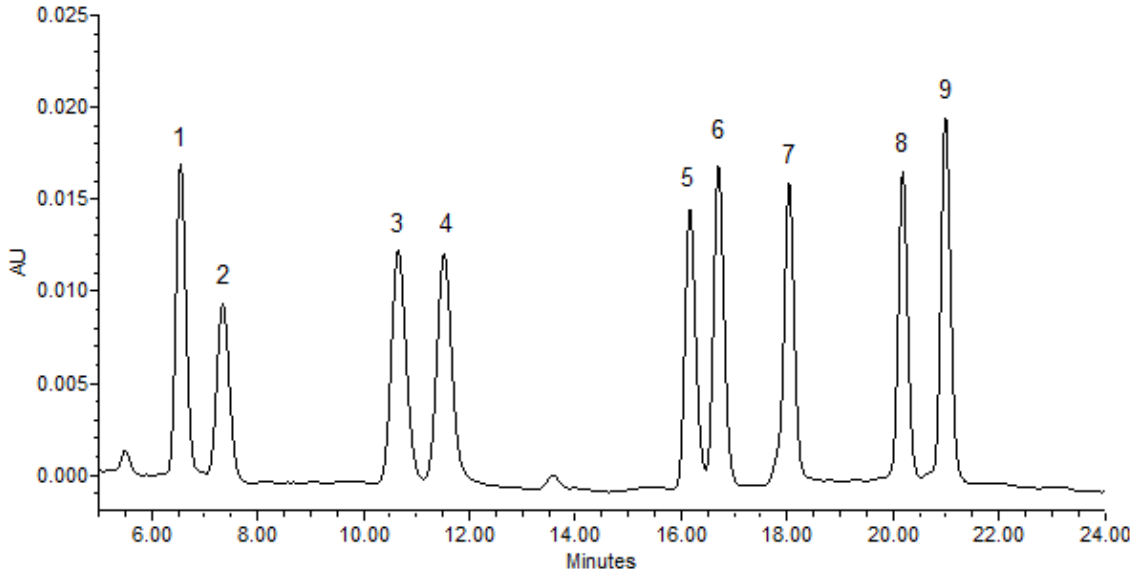


Figure 5. (a) MSPD marine sediment extract chromatogram and (b) MSPD spiked marine sediment extract chromatogram. Target compounds are numbered as follows: (1) simazine, (2) cyanazine, (3) simetryn, (4) atrazine, (5) ametryn, (6) propazine, (7) terbuthylazine, (8) prometryn, (9) terbutryn.

Table 1. Coefficient determination (R^2) and LODs and LOQs

Compound	Determination coefficient (R^2)	LOD (mg kg^{-1})	LOQ (mg kg^{-1})
Simazine	0.9999	0.007	0.025
Cyanazine	0.9998	0.011	0.037
Simetryn	0.9994	0.007	0.023
Atrazine	0.9992	0.007	0.024
Ametryn	0.9999	0.008	0.027
Propazine	0.9995	0.007	0.023
Terbuthylazine	0.9995	0.008	0.027
Prometryn	0.9994	0.008	0.026
Terbutryn	0.9999	0.007	0.022

Table 2. Precision and trueness of target compounds in sediment samples with MSPD-HPLC-DAD

Compound	Repeteability (%RSD, n=5)		Reproducibility (%RSD, n=6)		Accuracy (% Recovery, n=5)	
	0.1 mg kg^{-1}	1 mg kg^{-1}	0.1 mg kg^{-1}	1 mg kg^{-1}	0.1 mg kg^{-1}	1 mg kg^{-1}
Simazine	1.2	0.9	0.5	0.9	100.1	99.5
Cyanazine	3.4	1.4	2.8	1.9	96.1	99.1
Simetryn	1.6	2.3	1.6	2.0	99.5	96.7
Atrazine	1.3	2.4	1.0	2.1	100.4	98.8
Ametryn	1.7	2.0	1.5	2.3	101.4	98.9
Propazine	3.0	1.9	2.5	1.7	99.3	99.7
Terbuthylazine	1.1	2.0	1.3	0.5	95.0	99.8
Prometryn	1.2	2.1	1.6	3.3	99.8	99.9
Terbutryn	1.9	1.7	1.7	1.8	99.6	100.1

DEVELOPMENT OF A MATRIX SOLID PHASE DISPERSION METHODOLOGY FOR THE DETERMINATION OF TRIAZINE HERBICIDES IN MARINE SEDIMENTS

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INTRODUCTION

The contamination of the marine environment from herbicides is of great concern because of their high distribution in the aquatic system and of their toxic properties. Triazine herbicides are considered as an important class of chemical pollutants and European Union has included simazine and atrazine in the list of 33 priority substances in the EU Water Framework Directive (2000/60/EC) [1]. Moreover, the Directive 2008/105/EC [2] sets the Environmental Quality Standards (EQS) for these compounds in water and also shows the need to set EQS for these compounds in sediments and/or biota. Recently, terbutryn has been added to the list of priority substances by European Directive 2013/39/EU [3].

Sediments are considered to be compartments of accumulation of contaminants brought by the water column, therefore the analysis of sediments should be included in environmental studies because they are the result of the integration of all processes that occur in an aquatic ecosystem [4].

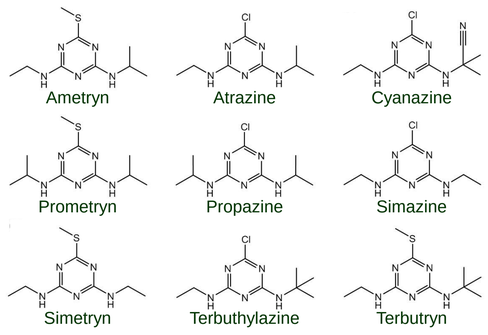
EXPERIMENTAL METHOD

HPLC-DAD Determination

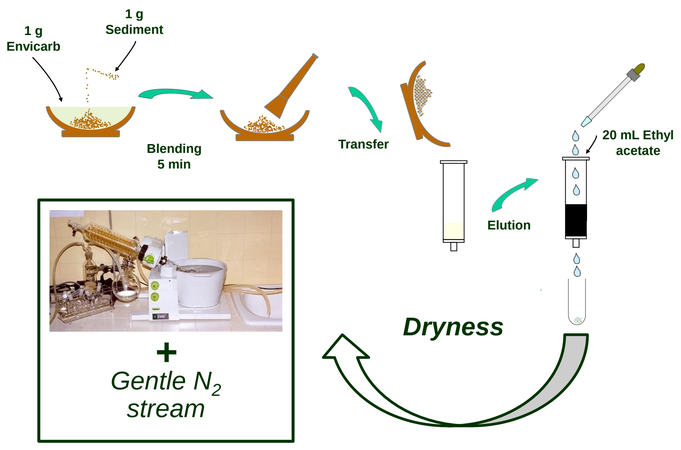
2695 pump with 996 DAD Waters
Hypersil GOLD C_{18} column
Absorbance: 222.7 nm

Gradient elution ACN:H_2O
Initial ACN 30% (8 min)
increased to 40% in 5 min
increased to 50% in 5 min
returned to initial conditions 9 min
Flow rate 1 mL min^{-1}
Sample volume: 20 µL

Triazines



MSPD Procedure



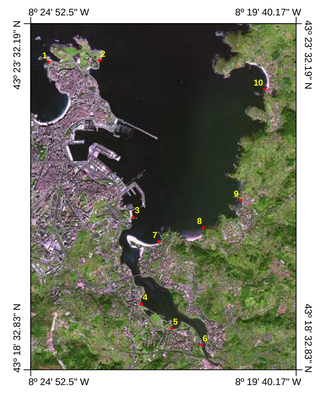
Residue was reconstituted in 1 mL methanol and the solution was filtered through of a 0.45 µm syringe filter of PTFE

Sample site location

Samples from 10 potential polluted sites (proximity to one or more potential sources of triazines).

Samples were homogenized, frozen (−20 °C) and freeze-dried. Then they were pulverized and sieved through a 0.5 mm mesh.

Estuary of Mero River, A Coruña



RESULTS AND DISCUSSION

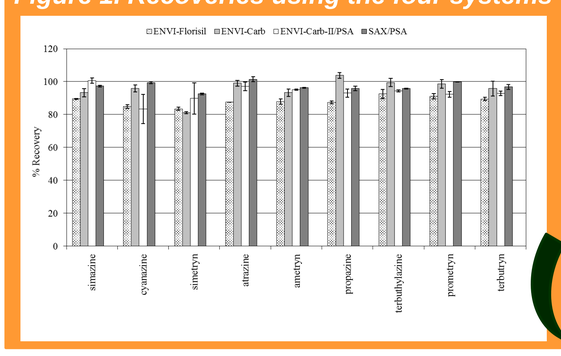
MSPD Optimization

Different experimental factors were tested:
extraction conditions (type of dispersant and clean-up co-sorbent) and elution solvent

Extraction optimization → 1 g of sediment spiked at the 1 mg kg^{-1} 20 and 5 mL of ethyl acetate acetonitrile as elution solvent

RP-C18 is the most used dispersant for extraction of triazines from plant and animal tissues [5]. Envi-18 (1 g) and four on-line clean-up co-sorbent were assayed (**see Fig.1**)

Figure 1. Recoveries using the four systems



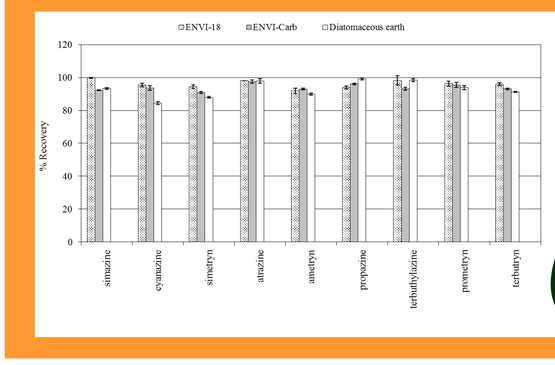
Eluates

ENVI-Florisil (1 g)
SAX/PSA (500 mg/500 mg)
ENVI-Carb-II/PSA (500 mg/500 mg)
ENVI-Carb (1 g)

Satisfactory recoveries (>80%)

Due to eluates with co-sorbents were clean enough to be injected, assays were carried out to check needless of a co-sorbent. Three dispersants (1 g) without clean-up step were evaluated (see **Fig. 2**)

Figure 2. Recoveries using three dispersants



Eluates

ENVI-18
Diatomaceous earth
ENVI-Carb

Satisfactory recoveries (>85%)

Elution → Acetonitrile and ethyl acetate are the most employed solvents for elution of triazines from MSPD systems [6]

- Elution was investigated collecting 5 eluates of 5 mL of above solvent
- 10 mL of ethyl acetate were necessary for recovering all compounds
- Symetrin required 20 mL of ethyl acetate
- Last fraction of acetonitrile contained non-detectable amounts of the target analytes

→ **20 mL of ethyl acetate as elution solvent were selected**

Method validation

The method was validated by estimation of the linearity, LODs and LOQs, recovery and precision
Results are presented in **Table 1 and 2**

Table 1. Linearity, LODs and LOQs

Compound	Determination coefficient (R^2)	LOD (mg kg^{-1})	LOQ (mg kg^{-1})
Simazine	0.9999	0.007	0.025
Cyanazine	0.9998	0.011	0.037
Simetryn	0.9994	0.007	0.023
Atrazine	0.9992	0.007	0.024
Ametryn	0.9999	0.008	0.027
Propazine	0.9995	0.007	0.023
Terbuthylazine	0.9995	0.008	0.027
Prometryn	0.9994	0.008	0.026
Terbutryn	0.9999	0.007	0.022

Table 2. Precision and trueness

Compound	Repeteability (%RSD, n=5)		Reproducibility (%RSD, n=6)		Accuracy (% Recovery, n=5)	
	0.1 mg kg^{-1}	1 mg kg^{-1}	0.1 mg kg^{-1}	1 mg kg^{-1}	0.1 mg kg^{-1}	1 mg kg^{-1}
Simazine	1.2	0.9	0.5	0.9	100.1	99.5
Cyanazine	3.4	1.4	2.8	1.9	96.1	99.1
Simetryn	1.6	2.3	1.6	2.0	99.5	96.7
Atrazine	1.3	2.4	1.0	2.1	100.4	98.8
Ametryn	1.7	2.0	1.5	2.3	101.4	98.9
Propazine	3.0	1.9	2.5	1.7	99.3	99.7
Terbuthylazine	1.1	2.0	1.3	0.5	95.0	99.8
Prometryn	1.2	2.1	1.6	3.3	99.8	99.9
Terbutryn	1.9	1.7	1.7	1.8	99.6	100.1

Analysis of estuarine sediment samples

The method was applied to the analysis of marine sediments from the estuary of Mero River (Galicia, NW of Spain). Although triazines were not detected, the analysis of these compounds is of great interest to control the quality of the marine ecosystem and to evaluate risks for human health since the presence of pesticides in sediments have been associated with a wide range of effects such as acute and chronic toxicity to aquatic organisms, such as algae, fish and shellfish.

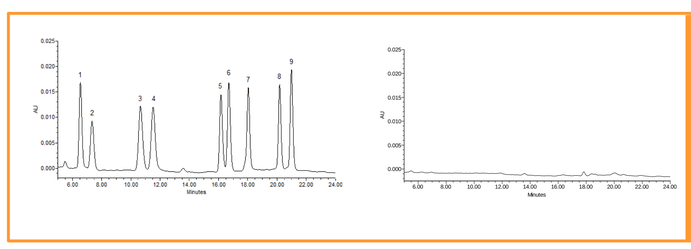


Figure 5
Chromatograms corresponding to unspiked and spiked sediment sample analysed under the considered procedure

CONCLUSIONS

- The suitability of a procedure based on MSPD for the extraction of nine triazines from sediment has been demonstrated for the first time. The method uses ENVI-Carb as dipersant and 20 mL ethyl acetate as elution solvent.
- The method provides satisfactory trueness and precision for the analysis of triazines in sediments.
- The main advantages of MSPD compared with classical methods of sample preparation are easy of work-up, fast, low cost, avoidance of clean-up procedure, as well as the significant reduction of organic solvents and energy requirements in agreement with the principles of the Green Chemistry.

Acknowledgments

This work has been financially supported by Xunta de Galicia potentially cofounded by European Regional Development Fund (EDRF) and by the Spanish Ministry of Economy and Competitiveness co-funded by ERDF (CTM2013-48194-C3-2-R). N. Rodríguez-González also thanks Xunta de Galicia for the concession of her Ph.D grant under the program of the International Campus of Excellence “Campus del Mar”

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DETERMINATION OF TRIAZINE HERBICIDES AND THEIR DEGRADATION PRODUCTS IN SEAWATER AND MARINE SEDIMENTS USING LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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ABSTRACT

Triazines and their degradation products are transported to the aquatic environment and, once there, the probability to reach the marine environment is very high. In this paper, solid phase extraction (SPE) and extraction by matrix solid phase dispersion (MSPD) to analyse nine triazines (ametryn, atrazine, cyanazine, prometryn, propazine, simazine, simetryn, terbuthylazine and terbutryn) and eight degradation products (desethylatrazine, desethyldesisopropylatrazine, desethyl-2-hydroxyatrazine, desethylterbuthylazine, desisopropylatrazine, desisopropyl-2-hydroxyatrazine, 2-hydroxyatrazine and 2-hidroxyterbuthylazine) in seawater and marine sediments samples were used. The analysis was carried out using liquid chromatography with tandem mass spectrometry (LC-ESI-MS/MS). The methods were optimized and validated to achieve a selective and sensitive determination of the analytes from different sample, regardless of its complexity. Under the optimum conditions, the proposed methods provided adequate limits of quantification (0.05-0.45 µg L^{-1} and 0.23-4.26 µg kg^{-1} in seawater and marine sediments respectively). Intra and inter-day relative standard deviation were below 1.41% for all compounds. Recoveries were evaluated and acceptable values ranged from 87.5-99.4% and 60.9-99.7% for the seawater and sediment samples respectively were obtained. The proposed methods were applied to the analysis of the target compounds in seawater samples and marine sediments from a coastal area of Galicia (NW of Spain).

Keywords: Triazines; Degradation products; Seawater; Sediments; Liquid chromatography; Mass spectrometry

1. Introduction

Triazines are a group of herbicides widely used to provide pre- and post-emergence of grasses, crops and many weeds in cereals (Wang et al. 2010). They are also employed for non-agricultural purposes including soil sterilization and road maintenance (Papadopoulos et al. 2012). However, it has been estimated that after their application, a

large proportion remains in the environment and due to their widespread use, persistence and mobility; fluxes of these compounds can reach the marine environment (Li et al. 2010). The cumulative effects of these compounds on the coastal environment are considerable (Camino-Sánchez et al. 2011).

The chemical contamination of the marine environment from herbicides is of great concern because of their high distribution in the aquatic system and their toxic properties; as an example, atrazine produces genotoxic damage in fish species (Santos and Martínez 2012). Triazines can be transformed in the environment by biotic and abiotic processes. Regarding their degradation products, not much information is available on their environmental impact, which can be even more toxic and persistent than parent compounds.

On one hand, triazine herbicides are considered an important class of chemical pollutants, hence included in the Endocrine Disruption Screening Program by the U.S. Environmental Protection Agency (2009). On the other hand, the European Union (EU) also included simazine and atrazine in the list of 33 priority substances in the EU Water Framework Directive (2000/60/EC) by way of Decision 2455/2001/EC. Moreover, the Directive 2008/105/EC sets the Environmental Quality Standards (EQS) for these compounds in surface water (2 and 4 µg L^{-1} for atrazine and simazine respectively) and committees the Member States to set EQS for these compounds in sediments and/or biota at national level. Recently, the Directive 2013/39/EU, amending the Directives 2000/60/EC and 2008/105/EC, includes terbutryn to the list of priority substances and establishes a maximum permitted concentration for this compound in surface water (0.34 µg L^{-1}). Although the EU Directive does not establish the maximum levels for the listed pollutants in sediments, the European Directive requires the Member States to monitor sediment at an adequate frequency to provide sufficient data of those priority substances that tend to accumulate in sediment.

Furthermore, Directive 2013/39/EU calls the attention on the important role of monitoring emerging pollutants that are not regularly considered in monitoring programs but can have toxicological effects. In this way, studies examining the concentration of triazines in surface waters have expanded the list of compounds including their main degradation products (hydroxy and dealkylated products) (Bottoni et al. 2013, Köck-Schulmeyer et al. 2012). Because of their mobility in the soil-water environment, the degradation products can reach marine environment more easily than triazines; thus the impact of herbicides tends to be underestimated when only the triazines are analysed in samples. Therefore, the main degradation products should be included in current analytical methods to obtain a better knowledge of aquatic ecosystem quality regarding herbicides contamination (Masiá et al. 2013).

Different chromatographic techniques have been used to determine triazines and/or their degradation products. Amongst these techniques, the application of liquid chromatography-tandem mass spectrometry (LC-MS/MS) has provided an increased selectivity and sensitivity (Beale et al. 2010, Dujakovic et al. 2010, García-Galán et al. 2010, Huff and Foster 2011, Kalogridi et al. 2014, Lissalde et al. 2001, Postigo et al. 2010, Zhang et al. 2014).

Regarding extraction procedure, solid phase extraction (SPE) is the preconcentration technique most commonly used for the determination of triazines and their degradation products in water samples. Nonpolar SPE sorbents are generally selected for extracting triazines from water samples. However, the degradation products, which contain polar functional groups, can be more efficiently extracted by using polar sorbents (Sabik et al. 2000). Different solid phases such as Oasis MCX cartridges (Li et al. 2013, Papadopoulos et al. 2007), PLRP-s (Hurtado-Sánchez et al. 2013, Postigo et al. 2010), Oasis HLB cartridges (Huff and Foster 2011, Hurtado-Sánchez et al. 2013), Amberlite XAD-4 resin (Akdogan et al. 2013) and Oasis MAX cartridges (Zhang et al. 2014) have been employed for triazines and their main degradation products. However, Oasis HLB has shown to have better ability to retain some degradation products (desethylatrazine, desisopropylatrazine, desethylterbuthylazine, 2-hydroxyatrazine and 2-hidroxyterbuthylazine) than other sorbents (Benvenuto et al. 2010, Gervais et al., 2008).

The most frequently used methodologies for the analysis of triazines in sediments employ solvent extraction procedures such as soxhlet (Galanopoulou et al. 2005), mechanical shaking (Li et al. 2010, Papadopoulos et al. 2012), sonication (Nödler et al. 2013), microwave-assisted extraction (MAE) (Fernández et al. 2013, Kalogridi et al. 2014) and pressurized liquid extraction (PLE) (Camino-Sánchez et al. 2011, Devault et al. 2010). In the last years, different innovation procedures have been developed and applied for the determination of pollutants in complex matrices with improved capabilities, reduced clean-up and concentration steps, the avoidance of toxic solvents and improved limits of detection. In this context, extraction techniques such as Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) (Brondi et al. 2011, Masiá et al. 2013) and matrix solid-phase dispersion (MSPD) (González-Mariño et al. 2010, Sánchez-Brunete et al. 2010) appear to be appropriate for the determination of emerging contaminants in sediments.

MSPD is an extraction procedure, which combines aspects of several analytical techniques allowing sample homogenization, disruption, extraction, fractionation and clean-up within a single process (Barker 2000). MSPD methods have been developed for extraction of contaminants from both vegetable and animal matrices. However, application of the MSPD methods to environmental solid samples such as soil or

sediments is relatively minimal compared to solvent extraction techniques (Capriotti et al. 2013).

Therefore, it is necessary to have accurate, effective, simple and fast methods of analysis for these compounds. Their determination in environmental matrices enable to improve the knowledge and data available on sources of these priority substances, and ways in which pollution occurs (Brondi et al. 2011).

The aim of this work was the development and validation of selective, sensitive and accurate analytical methodology for the quantification of 17 compounds (nine triazine herbicides as well as their main degradation products) in two compartments of the marine environment. The methods are based on SPE and MSPD using LC-ESI-MS/MS for simultaneous analyses of all compounds in seawater and marine sediment samples. Furthermore, the proposed methods were applied to the analysis of the target compounds in seawater and marine sediments samples from ten points susceptible to contamination by triazines from a coastline area of A Coruña (Galicia, NW of Spain).

2. Materials and methods

2.1. Chemicals and materials

All herbicides analytical standards were supplied by Sigma-Aldrich (Inc. St. Louis, MO, USA). The molecular structures of the target compounds are shown in Figure 1. The individual stock standard solutions of 100 mg L^{-1} were prepared in methanol by exact weighing of high-purity substances and stored at -18 ºC in the dark. Then, two working standard solutions (Mix I and II) of concentration 1 mg L^{-1} were prepared using methanol. Mix I contained the compounds having higher sensitivity (ametryn, atrazine, cyanazine, desethylterbuthylazine (DET), desethylatrazine (DEA), desisopropylatrazine (DIA), 2-hydroxyatrazine (HA), 2-hidroxyterbuthylazine (HT), prometryn, propazine, simazine, simetryn, terbuthylazine and terbutryn). Mix II contained desethyldesisopropylatrazine (DEDIA), desethyl-2-hydroxyatrazine (DEHA) and desisopropyl- 2-hydroxyatrazine (DIHA). All working solutions were freshly prepared every time by appropriate dilution of the mixed solution with methanol.

Acetonitrile (HPLC grade) and ethyl acetate (PAR, solvents for analysis of pesticide residues by GC) for instrumental analysis were purchased from Panreac (Barcelona, Spain). Methanol of superpurity was obtained from Romil (Cambridge, UK). Acetic acid (LC grade) was purchased from Sigma-Aldrich (Inc. St Louis, MO, USA). Milli-Q water was freshly prepared and obtained from a purification system from Millipore (Billerica, MA).

Oasis HLB cartridges (6 mL, 200 mg) were supplied by Waters (Milford, MA, USA) and ENVI-Carb bulk packing was from Sigma-Aldrich (Inc. St Louis, MO, USA).

Empty glass solid phase extraction syringes (6 mL capacity) and 20 µm polyethylene frits were purchased from Sigma-Aldrich (Inc. St Louis, MO, USA). Syringe polytetrafluoroethylene (PTFE) filters of 0.2 µm were obtained from Teknocroma (Barcelona, Spain).

A Visiprep® vacuum distribution manifold from Supelco (Bellefonte, PA, USA) was employed in the purification step. A Büchi R-3000 rotary-evaporator (Büchi Labortechnic AG, Flawil, Switzerland) was used in the evaporation step.

2.2. Sample collection

The area studied is located in the coastline of Galicia (NW of Spain). Seawater and marine sediment samples were collected from 10 potential polluted sites by triazines in estuary of A Coruña (see Fig. 1) during July of 2016. The mouth of Mero River forms the estuary and many small rivers and brooks that traverse areas dedicated to agriculture feed it. The sampling locations were selected by their proximity to one or more potential sources of contamination by triazine herbicides. The study area is illustrated in Fig. 1 and the 10 sampling points and the source in brackets are listed below: 1 (tram line), 2 (golf course), 3 (a railway line and vegetable gardens), 4 (vegetable gardens), 5 (mouth of a river flowing through a golf course and growing areas), 6 (mouth of the Mero river that traverses areas dedicated to agriculture), 7 and 8 (residential areas with gardens and parks), 9 (mouth of two rivers flowing through a campsite, vegetable gardens and residential areas with gardens) and 10 (lake with access to the sea which receives two rivers that cross growing areas, vegetable gardens, and residential areas with gardens). At each sampling point, three samples were collected.

Furthermore, unpolluted seawater samples from the Riazor beach at the city of A Coruña (Galicia, NW Spain) were used for the optimization and validation of the SPE procedure.

Seawater samples were collected in amber glass containers and transported to the laboratory under cooled conditions (4 ºC). Upon reception, samples were filtered through 0.6 µm glass fibre MN GF-6 filters from Macherey Nagel (Düren, Germany) to eliminate suspended solid matter. Due to the low stability of triazines, the samples were analysed the day of sampling.

Uncontaminated marine sediments collected from the Ares estuary (A Coruña, NW Spain) were used for optimization and validation of MSPD procedure. After collection, samples were homogenized, frozen at −20 °C and freeze-dried. The samples were then pulverized by using a vibrating ball mill and sieved through a 0.5 mm mesh. Finally, the samples were stored in amber glass bottles with hermetic seals out of light exposure until analysis.

2.3. Liquid chromatography-tandem mass spectrometry

The LC-ESI-MS/MS analyses were performed using an Agilent HP-1200 Series LC system equipped with an autosampler, a binary solvent pump and a thermostated column oven. The LC system was coupled to a mass spectrometer with a triple quadrupole detector (API 3200, Applied Biosystem, Carlsbad, CA, USA) equipped with an ESI source.

The column was a stainless steel column (150 mm x 4.6 mm ID, particle size 5 µm) packed with Hypersil GOLD C_{18} chemical bonded phase from Thermo Scientific (Austin, TX, USA). Elution was performed under gradient mode using acetonitrile as mobile phase A and 0.3% acetic acid in water as mobile phase B. Separation was carried out using a flow rate of 1 mL min^{-1}.

The gradient elution was performed as follows: mobile phase A initial percentage of 30% (2 min) increased linearly to 50% in 13 min, after which the percentage was returned to the initial conditions in 8 min. The sample volume injected into the LC system was 20 µL. The optimized method allowed the concurrent detection of 17 pesticides in a chromatographic run of 23 min.

The LC-ESI-MS/MS analysis of the herbicides was carried out in the positive ionization mode. Data acquisition was performed under multiple reaction monitoring (MRM) mode, recording the transition between the precursor ion and the two most abundant product ions for each target analyte. The optimization of MS/MS conditions, including the search for precursor and product ions, optimization of the sample cone voltage and collision energy was performed using the Analyst 1.4.2 software. MRM conditions used for analysis of the pesticides are shown in Table 1.

The ESI interface conditions were optimized for maximum intensity of the precursor ions as follows: capillary voltage 5 kV, source and desolvation temperature 600 °C. The nebulizer and desolvation gas flows were set at 850 and 50 L h^{-1} respectively. Nitrogen and air (35 psi) were used as nebulizer and desolvation gases. Nitrogen was used as collision gas at a pressure of 5 psi.

2.4 Extraction methods

In the case of seawater, an extraction method based on SPE using Oasis HLB cartridges was used. The extraction was performed by loading 10 mL of the sample solution at 10 mL min^{-1} through an OASIS® HLB cartridge previously conditioned with 10 mL of methanol and 10 mL of Milli-Q water. After sample loading, the cartridge was washed with 5 mL of Milli-Q water. Once the retention step had been completed, the cartridge was totally dried using a nitrogen stream for 30 min. Retained compounds were eluted with 2.5 mL of methanol. Finally, the eluate was filtered through a 0.2 µm PTFE filter.

The extraction method for sediments was based on MSPD. The extraction procedure was performed as follows: 1.0000 g of freeze-dried sediment sample was homogenised with 1 g of ENVI-Carb in a glass mortar with a pestle for 5 min. The final mixture was transferred into a 6 mL glass syringe and once packed, MSPD columns were connected to a Visiprep® vacuum distribution manifold. Elution was performed with 20 mL of ethyl acetate and 5 mL of acetonitrile. The obtained eluate was evaporated to a drop in rotary-evaporator and got to dryness by a gentle nitrogen stream. The residue was reconstituted in 1 mL methanol and the solution was filtered through of a 0.2 µm syringe filter of PTFE.

3. Results and discussion

3.1. LC-MS/MS development

The optimum cone voltage and collision energy for each pesticide was selected with the aim of obtaining the precursor ion and the MRM transition with the highest sensitivity and the other product ion (Table 1). The most sensitive transition was chosen for quantification and the other transition was used for confirmation. Optimization of MS/MS settings was performed by direct infusion of individual standard solutions (0.5-1 µg mL^{-1} in methanol). Negative or positive mode was studied for all analytes. The analyses of the compounds were performed in the positive mode. The suitable settings of the instrument for each pesticide are shown in Table 1.

The LC method was optimized to get a good peak separation. The optimization was performed using standard solutions of 1 µg mL^{-1} in methanol. Since degradation products are strongly dependent on pH, the use of a mobile phase with a buffer or a modifier is necessary. The most employed mobile phases with modifiers are acetonitrile-acetic acid solution (Gikas et al., 2012), acetonitrile-ammonium formate and acetonitrile-formic acid (Li et al., 2013). For this reason, these modifier solutions as mobile phase B were studied, and the best chromatographic separation was obtained using acetonitrile-acetic acid solution.

Once chosen the modifier, the concentration of acetic acid in the mobile phase B was assayed in order to achieve optimum separation. Three concentration of acetic acid (0.1, 0.3 and 0.5%) were evaluated. The best chromatographic separation was achieved with 0.3% of acetic acid, whereas a higher overlap was observed with 0.5% and four compounds were missing when employing 0.1%. Figure 2 shows a MS chromatogram employing acetonitrile-0.3% acetic acid as mobile phase. From the chromatogram, it is evident that some compounds remain overlapped. However, the use of tandem MS enabled an accurate analysis even of co-eluted compounds.

3.2. Extraction methods

3.2.1. Extraction methods performance

Both extraction methods are based on procedures previously developed by the authors for the analysis of the nine target triazines in seawater and marine sediments using DAD as detection system (Rodríguez-González et al. 2013, 2016). For this reason, it was necessary to modify both methods since in this work, the analysis of their main degradation products was included and a MS/MS detector was employed.

Regarding SPE method, the sample volume was reduced from 25 to 10 mL due to the high sensitivity of mass detector. Furthermore, Milli-Q volume used for washing the loaded cartridge was decreased from 20 to 5 mL to avoid loss of the more polar degradation products.

On the other hand, in order to remove the concentration step of the eluate using rotary-evaporator and simplify the method, acetone was replaced by methanol (2.5 mL) as elution solvent. For this purpose, it was taken into account the results obtained by Beceiro-González et al. (2014) which have shown satisfactory recoveries using different elution solvents (acetonitrile, methanol and acetone) for the nine triazine studied.

In the case of MSPD method, due to higher polarity of the degradation products, the eluent was slightly modified by adding 5 mL of acetonitrile to the eluent of the initial method (20 mL of ethyl acetate). This modification was based on a previous study for the determination of target triazines in seaweeds (Rodriguez-González et al. 2014).

In this work, volume and type of elution solvent (ethyl acetate, acetonitrile and mixtures of both solvents) were optimized and the best results were obtained using sequential elution of 20 mL ethyl acetate and 5 mL acetonitrile.

3.2.2. Methods Validation

Both methods were validated in terms of linearity, limits of detection and quantification, precision and accuracy. The limits of detection (LODs) were calculated as $3*S_{y/x}/b$ and the limits of quantification (LOQs) as $10*S_{y/x}/b$, where $S_{y/x}$ is the residual standard deviation and b is the slope of the matrix calibration curves. The precision of the overall analytical procedures, expressed as relative standard deviation (RSD), was evaluated as intra-day and inter-day precision. To study intra-day precision, RSDs were calculated by spiking samples at two levels of concentrations (one level close to LOQ of each compound and the other five times higher) measuring five replicates of spiked samples at each concentration level during the same day. The inter-day precision was studied at an intermediate level of concentration by measuring five replicates on three consecutive days. Finally, the accuracy of the methods was evaluated in terms of recovery.

SPE method: The linearity was evaluated using standard solutions analysed with the proposed method. Calibration curves were constructed using six calibration points, with three replicates for each calibration level, at the concentration range shown in Table 2. As it can be seen, very good linearity was obtained for all the compounds with coefficients of determination (R^2) higher than 0.996.

LODs and LOQs were in the range of 0.02-0.03 and 0.05-0.10 µg L^{-1} respectively for all compounds, except DEDIA, DEHA and DIHA (Table 2). These values were satisfactory because LOQs are lower than 30% of the more restrictive parametric value requested by the legislation for triazines in surface water (maximum permitted concentration of 0.34 µg L^{-1} for terbutryn). For DEDIA, DEHA and DIHA, the limits of detection and quantification (between 0.08-0.15 and 0.26-0.45 µg L^{-1} respectively) were adequate, being the LOQs < 30% of the parametric value requested by the legislation for atrazine in surface water (Directive 2013/39/EU). LOQs obtained permit to ensure proper determination of the target compounds at the levels established by the Directive 2013/39/UE. Moreover, the selectivity of the method was evaluated by analysing control blank samples. The absence of any signal at the same retention time of the selected compounds indicated there were no any matrix interferences or contamination that can give a false positive signal.

Regarding precision, the results are shown in Table 3. The values obtained for intra-day and inter-day precision were satisfactory for all compounds (RSDs lower than or equal to 1.41 %) indicating that the developed method was reproducible. Accuracy of the proposed method was determined by spiking 10 mL of unpolluted seawater sample at two levels of concentration depending on the compound (0.25 and 1.25 µg L^{-1} for DEDIA, DEHA and DIHA and 0.05 and 0.25 µg L^{-1} for the remaining compounds). Five replicates at each fortification level were used and the analytical recoveries of the spiked samples were calculated. As it can be seen in Table 3, satisfactory recoveries at both levels of concentration for all compounds were achieved. Figure 3 shows the MS/MS chromatograms of the degradation products and four triazines for a seawater sample spiked at 0.05 and 0.25 µg L^{-1} depending on the compound.

MSPD method: The matrix effect was evaluated since signal suppression or enhancement can severely compromise quantitative analysis of the compounds at trace levels. The matrix effect was studied by comparison of the slopes of the calibration curves in solvent and in extracts of blank sediments obtained after the MSPD procedure (employing six points). Both the solvent and the matrix calibration curves had good linearity, with determination coefficients higher than 0.999 for solvent calibration curves and for 0.998 for matrix-matched ones. Most of compounds did not show matrix

effect, except of cyanazine and DEHA, which evidenced a light matrix effect and signal suppression.

The linearity was evaluated by spiking sediment samples with different amounts of the target compounds and then analysed with the proposed method. Calibration curves were constructed using six calibration points, with three replicates for each calibration level, at a concentration range shown in Table 2. As can be seen, good linearity was obtained for all compounds with coefficients of determination (R^2) higher than 0.997.

LODs and LOQs obtained for all compounds (between 0.08-1.40 and 0.23-4.26 µg kg^{-1} respectively) were adequate. Although there is no legislation for these compounds in sediments, the LOQs obtained (Table 2) were satisfactory because the values for all compounds (except DEDIA, DEHA and DIHA) were 20-40 times lower than the maximum limit of the most restrictive parametric value requested by the European legislation for triazines in edible seaweed (10 µg kg^{-1} for simazine) (Commision Regulation (EC) No 310/2011). For DEDIA, DEHA and DIHA, the LOQs obtained were 10-20 times lower than the maximum limit established by legislation for terbuthylazine in edible seaweed (50 µg kg^{-1}) (Commision Regulation (EC) No 149/2008).

The values obtained for intra-day and inter-day precision are shown in Table 3. The RSD values were lower than 0.73 % (Table 3) which indicate the developed method was reproducible. Accuracy was determined by spiking 1.0000 g of uncontaminated marine sediment sample at two concentration levels (0.5 and 2.5 µg kg^{-1} for ametryn, atrazine, cyanazine, DEA, DET, DIA, HA, HT, prometryn, propazine, simetryn, simazine, terbutryn and terbuthylazine and 5 and 25 µg kg^{-1} for DEDIA, DEHA and DIHA). To evaluate the accuracy, the analytical recoveries of spiked samples, using five replicates at each fortification level, were calculated using matrix-matched calibration standards. The analytical recoveries of the seventeen analytes were acceptable for both levels of concentration (between 60.9 and 99.7 %). Figure 4 shows the MS/MS chromatograms of a marine sediment spiked at 0.5 µg kg^{-1} level corresponding to atrazine, prometryn, propazine, terbutryn and terbuthylazine.

3.3. Application of the method to the analysis of seawater and marines sediments samples

The proposed methods were applied to analyse the target compounds in seawater and marine sediment samples from ten points of the coastline area of Galicia (NW of Spain). The sampling took place during July of 2016. The seawater samples registered concentrations of triazines and degradation products below the LODs. In the case of sediments, out of the 17 compounds analysed only terbuthylazine was detected in sample M4 (0.45 µg Kg^{-1}).

4. Conclusions

The analysis of triazines and their degradation products in the marine environment is of great interest in order to control the quality of the marine environment and to evaluate risks for human health. The presence of pesticides in seawater and sediments has been associated with a wide range of effects such as acute and chronic toxicity to aquatic organisms. These compounds as well as others emerging contaminants have been the goal of the development of methods in order to find viable determination procedures that could be applied to complex and diluted matrices.

The proposed methods based on SPE and MSPD coupled to LC-ESI-MS/MS have been demonstrated to be selective and sensitive for the simultaneous analysis of nine triazines herbicides and eight degradation products in seawater and marine sediments samples. An important difference of the proposed methods with previously described methodology for the analysis of triazines herbicides and their main degradation products is the determination of a greater number of degradation products simultaneously with triazines. It is noteworthy that methods based on solid-phase extraction combined with LC-MS/MS have been used to measure triazines and degradation products in river waters; however, there are not studies in seawater. Regarding sediments, to the best of our knowledge, no studies using MSPD have been published to extract these chemicals residues from sediments.

With the proposed analytical methodologies, satisfactory precision and accuracy were obtained for both methods. The limits of quantification achieved for seawater enable the determination of these pollutants at the levels required by European Union legislation (Directive 2013/39/EU). Although there is no legislation for these compounds in sediments, the LOQs obtained are much lower than the maximum limits legislated for triazines in edible seaweeds (Commision Regulation (EC) No 310/2011). Consequently, these methods can be an important tool to determine triazines and their degradation products at trace levels in marine environment.

Once optimized the methodology, we applied both methods to determine the target compounds in ten different seawater and marine sediments samples from the coastline of A Coruña (NW, Spain). Only one of the compounds was detected in one of the marine sediment analyzed.

It is worthy to note that both methods are simple, fast, with a lower consumption of solvents and energy requirements according to the principles of Green Chemistry.

Acknowledgements and funding

This work was funded by Ministerio de Economía y Competitividad (CTM2013-48194-C3-2-R) and Program of Consolidation and Structuring of Units of Competitive

Investigation of the University System of Galicia (Xunta de Galicia) potentially cofounded by European Regional Development Fund (ERDF) in the frame of the operative Program of Galicia 2007-2013 (reference: GRC2013-047).

N. Rodríguez-González has received a PhD grant from "Xunta de Galicia" and Campus do Mar (International Campus of Excellence).

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Figure. 1 Location of sampling sites in estuary of A Coruña (Galicia, NW of Spain)

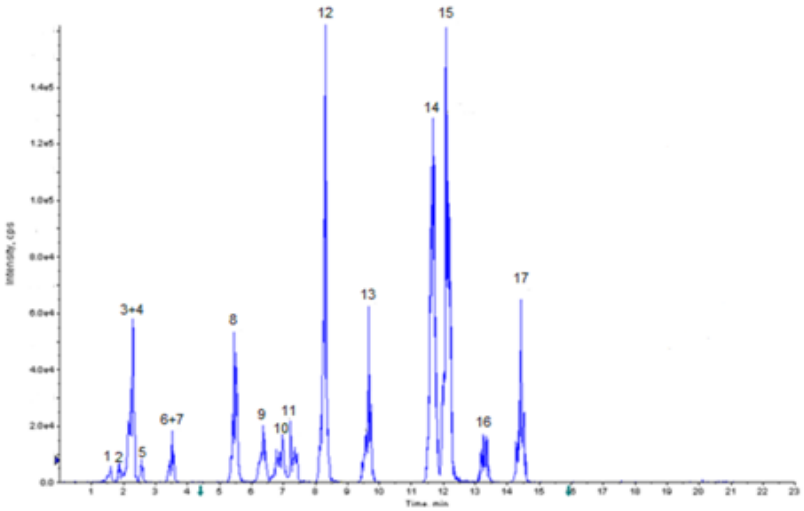

Figure 3. LC-MS chromatogram of standard mixture (1 µg mL^{-1} of each compound). Target compounds are numbered as follows: (1) DIHA, (2) DEHA, (3) HA, (4) DEDIA, (5) HT, (6) DEA, (7) DIA, (8) simetryn, (9) simazine, (10) cyanazine, (11) DET, (12) ametryn, (13) atrazine, (14) prometryn, (15) Terbutryn

(a)

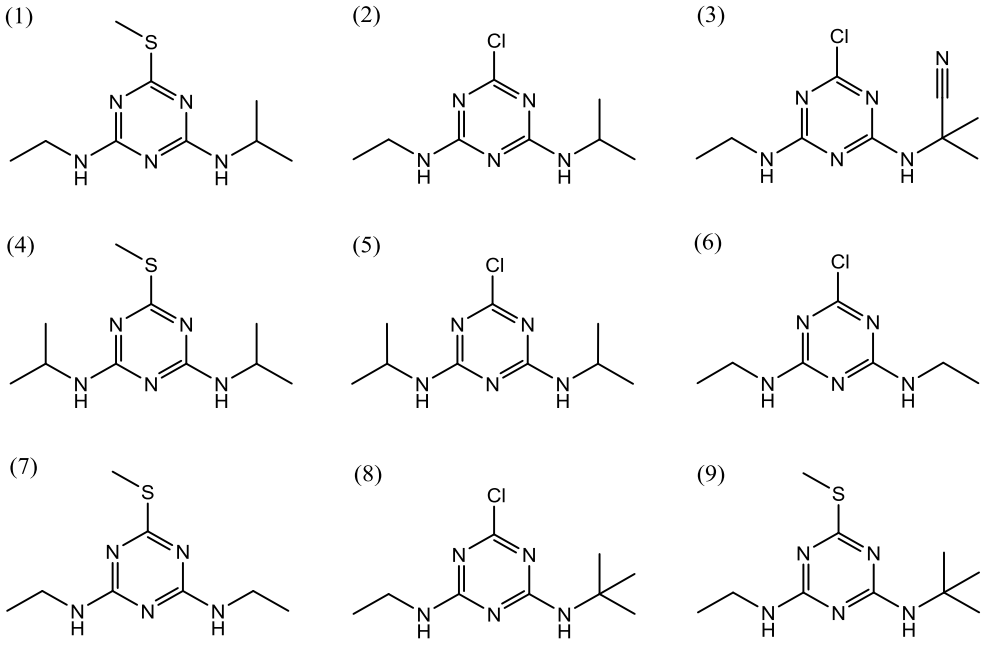

Figure 2. Structures of the target compounds. (a) Triazines: (1) Ametryn, (2) Atrazine, (3) Cyanazine, (4) Prometryn, (5) Propazine, (6) Simazine, (7) Simetryn, (8) Terbuthylazine and (9) Terbutryn

(b)

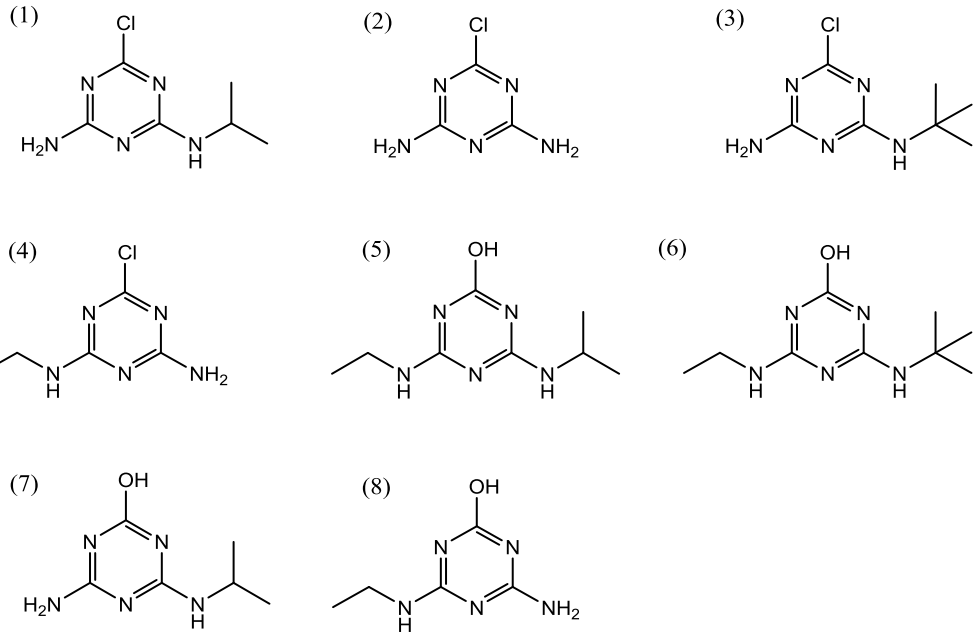

Figure 2. Structures of the target compounds. (b) Degradation products: (1) DEA, (2) DEDIA, (3) DET, (4) DIA, (5) HA, (6) HT, (7) DEHA and (8) DIHA

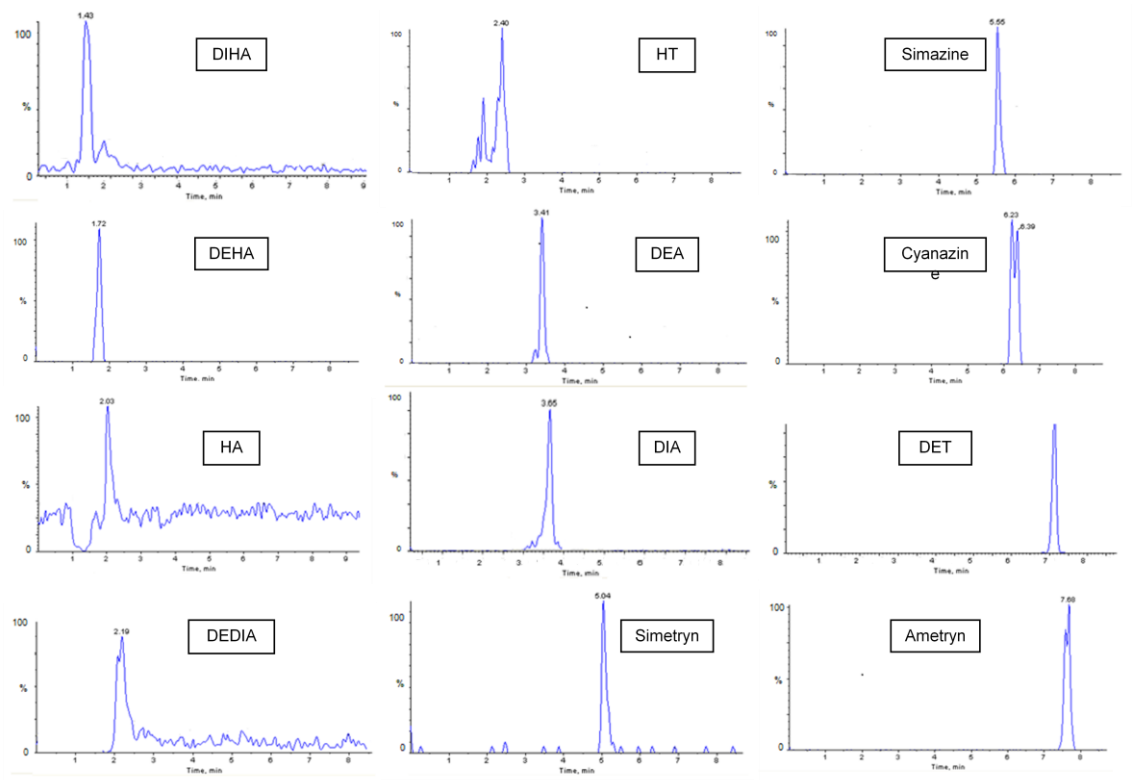

Figure 4. LC-MS/MS chromatograms of the degradation products and four triazines from a seawater sample spiked with 0.25 µg L^{-1} for DEDIA, DEHA and DIHA and 0.05 µg L^{-1} for remaining compounds

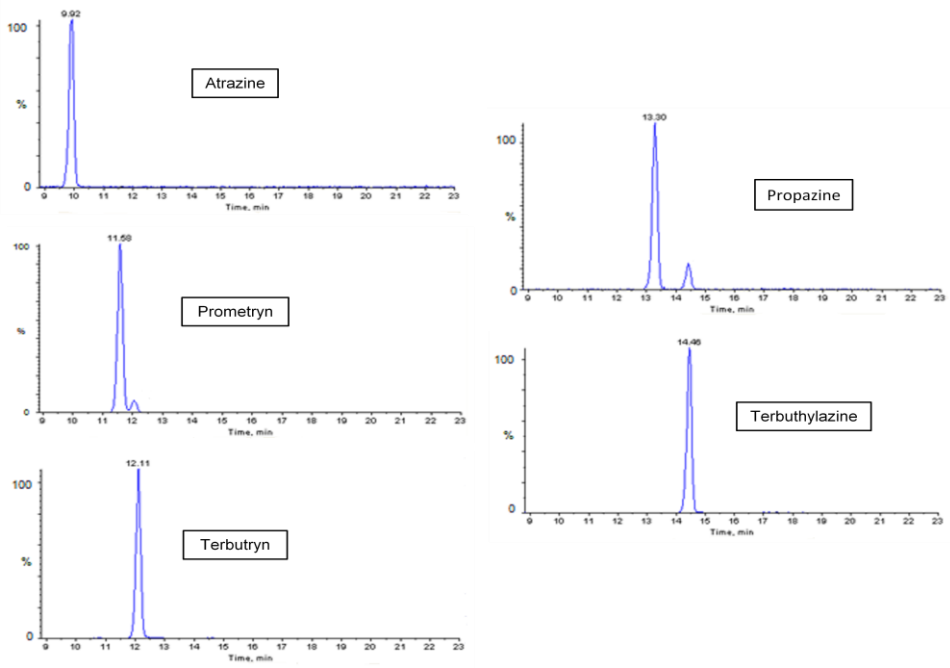

Figure 5. LC-MS/MS chromatograms of five triazines obtained after MSPD method was applied, from a marine sediment spiked with 0.5 µg kg^{-1} for each compound

Table 1. Retention time and MS/MS optimized parameters for the studied pesticides

Compound	Rt (min)	Precursor ion (m/z)	Product ion (m/z)	Cone (V)	Collision energy (eV)
Ametryn	8.47	228	186	8.000	23.000
			68	8.000	51.000
Atrazine	10.81	216	174	6.500	23.000
			68	6.500	49.000
Cyanazine	7.55	241	214	6.000	21.000
			68	6.000	53.000
DEA	3.73	188	146	12.000	25.000
			104	12.000	31.000
DEDIA	2.23	146	68	7.500	31.000
			62	7.500	49.000
DEHA	1.87	170	128	2.500	23.000
			86	2.500	31.000
DET	8.16	202	146	5.000	19.000
			104	5.000	35.000
DIA	2.72	174	68	9.000	37.000
			104	9.000	33.000
DIHA	1.72	156	70	11.000	37.000
			86	11.000	25.000
HA	2.23	198	69	5.500	43.000
			151	5.500	23.000
HT	2.51	212	156	5.500	19.000
			70	5.500	49.000
Prometryn	12.43	242	158	4.000	33.000
			68	4.000	51.000
Propazine	14.67	230	146	11.000	33.000
			58	11.000	37.000
Simazine	6.81	202	104	4.500	33.000
			68	4.500	45.000
Simetryn	5.32	214	68	6.000	47.000
			124	6.000	29.000
Terbuthylazine	15.79	230	174	4.500	21.000
			68	4.500	49.000
Terbutryn	12.51	242	186	4.500	25.000
			68	4.500	55.000

Table 2. Limits of detection and quantification of the LC-MS/MS methods

Compound	SPE method				MSPD method			
	Concentration range (μg L^{-1})	LOD (μg L^{-1})	LOQ (μg L^{-1})	Determination coefficient (R^2)	Concentration range (μg kg^{-1})	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	Determination coefficient (R^2)
Ametryn	0.025-0.5	0.02	0.05	0.9993	0.25-2.5	0.010	0.032	0.9989
Atrazine	0.025-0.5	0.02	0.07	0.9988	0.25-2.5	0.08	0.26	0.9993
Cyanazine	0.025-0.5	0.03	0.09	0.9981	0.25-2.5	0.08	0.24	0.9994
DEA	0.025-0.5	0.03	0.08	0.9983	0.25-2.5	0.13	0.38	0.9985
DEDIA	0.25-2	0.15	0.45	0.9961	2.5-25	1.04	3.16	0.9989
DEHA	0.25-2	0.09	0.29	0.9985	2.5-25	1.40	4.26	0.9981
DET	0.025-0.5	0.03	0.08	0.9984	0.25-2.5	0.10	0.31	0.9990
DIA	0.025-0.5	0.03	0.08	0.9983	0.25-2.5	0.15	0.45	0.9978
DIHA	0.25-2	0.08	0.26	0.9988	2.5-25	0.78	2.35	0.9994
HA	0.025-0.5	0.03	0.08	0.9982	0.25-2.5	0.09	0.28	0.9992
HT	0.025-0.5	0.02	0.07	0.9987	0.25-2.5	0.11	0.33	0.9989
Prometryn	0.025-0.5	0.02	0.05	0.9993	0.25-2.5	0.08	0.23	0.9994
Propazine	0.025-0.5	0.02	0.07	0.9988	0.25-2.5	0.15	0.46	0.9977
Simazine	0.025-0.5	0.02	0.07	0.9989	0.25-2.5	0.11	0.32	0.9989
Simetryn	0.025-0.5	0.03	0.10	0.9977	0.25-2.5	0.13	0.40	0.9983
Terbuthylazine	0.025-0.5	0.03	0.08	0.9985	0.25-2.5	0.13	0.40	0.9983
Terbutryn	0.025-0.5	0.03	0.09	0.9980	0.25-2.5	0.08	0.24	0.9994

Table 3. Precision and accuracy of the LC-MS/MS methods

Compound	SPE method					MSPD method				
	Intra-day precision RSD (%)[a]		Inter-day precision RSD (%)[b]	Recovery (%)		Intra-day precision RSD (%)[c]		Inter-day precision RSD (%)[d]	Recovery (%)	
	Level 1	Level 2		Level 1	Level 2	Level 1	Level 2		Level 1	Level 2
Ametryn	0.22	0.54	0.32	99.1	97.7	0.28	0.66	0.29	99.6	98.4
Atrazine	0.70	1.37	0.20	92.7	95.3	0.09	0.16	0.11	95.7	98.3
Cyanazine	1.41	0.21	0.02	97.5	98.1	0.13	0.27	0.14	96.9	98.4
DEA	0.12	0.29	0.30	97.4	97.9	0.56	0.10	0.50	96.2	98.2
DEDIA	0.38	0.15	0.36	87.5	91.0	0.71	0.22	0.73	76.6	77.1
DEHA	0.24	0.36	0.18	89.0	90.3	0.10	0.23	0.10	61.2	61.5
DET	0.81	0.82	0.68	97.7	96.0	0.15	0.29	0.17	95.1	97.7
DIA	0.70	0.15	0.22	94.4	98.1	0.33	0.62	0.36	92.4	93.6
DIHA	0.65	0.20	0.23	91.2	93.7	0.26	0.61	0.34	97.2	96.9
HA	0.59	0.21	0.05	89.4	93.1	0.48	0.09	0.49	60.9	61.0
HT	0.19	0.41	0.18	89.4	91.3	0.30	0.52	0.30	89.2	90.1
Prometryn	0.33	0.59	0.06	98.1	98.4	0.66	0.44	0.63	96.1	97.7
Propazine	0.49	0.78	1.06	98.4	97.9	0.15	0.23	0.09	97.3	99.7
Simazine	0.61	0.57	0.92	93.5	95.5	0.16	0.42	0.17	97.7	94.4
Simetryn	0.38	0.36	0.94	98.8	98.6	0.63	0.01	0.65	89.0	90.8
Terbuthylazine	0.25	0.80	0.27	98.7	98.1	0.53	0.11	0.60	97.1	94.6
Terbutryn	1.13	0.26	0.17	96.0	99.4	0.16	0.19	0.17	97.7	99.3

[a] 0.05 and 0.25 µg L^{-1} for the nine triazines, DEA, DET, DIA, HA and HT and 0.25 and 1.25 µg L^{-1} for DEDIA, DEHA and DIHA

CAPÍTULO IV

BIOTA

INTRODUCCIÓN

En el capítulo IV, se desarrollan nuevos métodos analíticos para la determinación de triazinas en distintas matrices de biota (alga, mejillón y trucha), que permiten cuantificar los residuos de estos compuestos dentro de los niveles permitidos por la legislación Europea. Para cada método propuesto se recoge tanto la optimización de cada una de las distintas etapas (extracción, purificación y concentración), como el estudio de validación. Por último, para cada tipo de matriz, se realiza la aplicación a muestras reales y se comentan las ventajas y aportaciones respecto a los métodos previamente publicados.

Los métodos desarrollados se basan en la extracción de las triazinas mediante dispersión de la matriz en fase sólida, seguido de la limpieza del extracto mediante extracción en fase sólida y empleando para la determinación de los nueve herbicidas triazínicos (Ametrina, Atrazina, Cianazina, Prometrina, Propazina, Simazina, Simetrina, Terbutilazina y Terbutrina) cromatografía de líquidos de alta resolución con detección de red de diodos.

En primer lugar, se llevó a cabo el desarrollo y validación de un método de MSPD para la determinación de las triazinas en muestras de alga. Para la optimización se empleó un alga verde (lechuga de mar). El adsorbente elegido para la extracción de las triazinas es C_8, que es una fase enlazada lipófila indicada para analitos de polaridad intermedia, y como coadsorbentes se emplearon ENVI-Carb II/PSA. Con respecto al disolvente de elución se ensayaron dos eluyentes (acetato de etilo y acetonitrilo) de los cuales se probaron mezclas en distintas proporciones de cada uno, y distintos volúmenes de elución, obteniéndose los mejores resultados empleando 20 mL de acetato de etilo y 5 mL de acetonitrilo. Una vez optimizado y validado el método, se aplicó al análisis de muestras de algas rojas (Nori) y pardas (Wakame), obteniéndose resultados satisfactorios.

Posteriormente, se optimizó y validó un método de MSPD para muestras de mejillón. Se estudiaron dos agentes dispersantes (C_{18} y florisil) y se probaron ocho coadsorbentes para la etapa de limpieza (florisil, sílica, sílica/alúmina, Envi-Carb, Envi-Carb-II/PSA, SAX/PSA, Envi-Carb-II/SAX/PSA y C_{18}). Los mejores resultados se consiguieron empleando C_{18} como agente dispersante y cartuchos de triple capa Envi-Carb-II /SAX/PSA como coadsorbentes. Como eluyente se empleó una elución secuencial con 20 mL de acetato de etilo y 5 mL de acetonitrilo. Por último, el método se aplicó a diferentes muestras de mejillón adquiridas en un supermercado local de A Coruña.

El método desarrollado para la determinación de triazinas en muestras de mejillón, se aplicó a muestras de trucha de acuicultura. Debido al elevado contenido

graso de la matriz, fue necesario disminuir la cantidad de muestra, manteniendo la misma cantidad de agente dispersante C_{18}, los mismos cartuchos de coadsorbente y los mismos disolventes para la elución de las triazinas. La determinación de las triazinas se llevó a cabo mediante HPLC-DAD y se realizó su confirmación mediante LC-ESI-MS/MS. Las truchas empleadas para el estudio fueron adquiridas en una pescadería local de A Coruña.

Publicaciones:

Determination of triazine herbicides in seaweeds: development of a sample preparation method based on matrix solid phase dispersion and solid phase extraction clean-up. (2014). Rodríguez-González, N., González-Castro, M.J., Beceiro-González, E., Muniategui-Lorenzo, S., Prada-Rodríguez, D. *Talanta*. 121, 194-198. **Anexo I-IV, página 313.**

Development of a matrix solid phase dispersion methodology for the determination of triazine herbicides in mussels. (2015). Rodríquez-González, N., González-Castro, M.J., Beceiro-Gonzalez, E., Muniategui-Lorenzo, S. *Food Chemistry*. 173, 391-396. **Anexo I-V, página 321.**

Validation of a matrix solid phase dispersion methodology for the determination of triazines herbicides in fish. (2015). González-Castro, M.J., Castro-Bustelo, V., Rodríguez-González, N., Beceiro-González, E. *Advances in Food Analysis Research, Nova Science Publishers.* 8, 167-176. **Anexo I-VI, página 329.**

DETERMINATION OF TRIAZINE HERBICIDES IN SEAWEEDS: DEVELOPMENT OF A SAMPLE PREPARATION METHOD BASED ON MATRIX SOLID PHASE DISPERSION AND SOLID PHASE EXTRACTION CLEAN-UP

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ABSTRACT

A method using dual process columns of Matrix Solid Phase Dispersion (MSPD) and Solid Phase Extraction (SPE) has been developed for extracting and cleaning-up of nine triazine herbicides (ametryn, atrazine, cyanazine, prometryn, propazine, simazine, simetryn, terbuthylazine and terbutryn) in seaweed samples. Under optimized conditions, samples were blended with 2 g of octasilyl-derivatized silica (C_8) and transferred into a SPE cartridge containing ENVI^{TM}-Carb II/PSA (0.5/0.5 g) as clean up co-sorbent. Then the dispersed sample was washed with 10 mL of *n*-hexane and triazines were eluted with 20 mL ethyl acetate and 5 mL acetonitrile. Finally the extract was concentrated to dryness, re-constituted with 1 mL methanol:water (1:1) and injected into the HPLC-DAD system. The linearity of the calibration curves was excellent in matrix matched standards, and yielded the coefficients of determination $\geq$ 0.995 for all the target analytes. The recoveries ranged from 75-100% with relative standard deviations lower than 7%. The achieved LOQs (<10 $\mu g kg^{-1}$) for all triazines under study permits ensure proper determination at the maximum allowed residue levels set in the European Union legislation. Samples of three seaweeds were subjected to the procedure proving the suitability of MSPD method for the analysis of triazines in different seaweeds samples.

Keywords: Triazine herbicides. Seaweeds. Matrix solid phase dispersion. Solid phase extraction. High performance liquid chromatography. Diode array detection.

1. Introduction

Seaweeds have been used since ancient times as food, fodder, fertilizer and as a source of medication. Nowadays, seaweeds are the raw material for industrial production of agar, carrageenan and alginates, however they still remain to be widely consumed as a source of food in Asian countries. Although in North America and Europe their uses as food is more restricted, in recent years seaweeds have been increasingly recognized as healthy and attractive foods [1]. Edible seaweeds contain dietary fibre, high

concentration of minerals, vitamins, proteins, polyunsaturated fatty acids and low content in saturated fats. On the other hand, seaweeds have shown also biological properties such as antibacterial, antiviral, antioxidant and antifungal [2,3]. Moreover, it has been reported that the chemical composition of seaweeds varies with species, habitats, maturity and environmental conditions [4].

Triazines, well-known herbicides, are applied to soil for the control of weeds in many agricultural crops, as well as, railways, roadside and golf courses. The marine environment receives fluxes of these compounds mainly from agricultural origin. Their mechanism of action is via photosynthetic inhibition, and for this reason, they are only toxic for plants; however these compounds can affect the human health through the dietary intake. These compounds are highly persistent and can survive many years in soil, water and organisms. Therefore, they are considered as an important class of chemical pollutants and atrazine and simazine have been included in the group of endocrine-disrupting by the Environmental Protection Agency of US [5]. As a result, the European Parliament and Council [6] concerning the residue levels on food and feed of plant and animal origin establishes as maximum permitted concentration in seaweed 0.01 and 0.05 mg kg^{-1} for simazine and terbuthylazine respectively. Moreover, a limit is not yet established for atrazine in seaweed, but its maximum permitted limit in edible vegetables is 0.05 mg kg^{-1}. For this reason, analytical methods for a rapid and sensitive determination of these compounds are required. However, seaweed is a complex matrix with different types of interfering compounds which make pesticide analysis difficult; in fact, studies of pesticides in seaweeds are limited and recent [7-9] and to the best of our knowledge there is only one reference in the literature devoted to the determination of triazines in seaweeds [10].

The more frecuently used methodologies for the analysis of triazines in samples of vegetable and animal origin employ solvent extraction procedures such as soxhlet [11,12] shake flask [13,14], sonication [10,13,15], microwave-assisted extraction (MAE) [11,12,16,17], and pressurized liquid extraction (PLE) [18,19]. Nevertheless, they generally need to add a clean-up step to decrease the presence of interferents in the final extract to reduce the detection limits of the methods and to avoid inaccurate results in the chromatographic determination, which is time consuming, many times expensive and simultaneously decreases the precision of the methodologies involved. In the last years, different innovative procedures have been developed and applied for the determination of pollutants in complex matrices with improved capabilities, reduced clean up and concentration steps, the avoidance of toxic solvents and improved limits of detection. In this context, sorptive extraction techniques like solid-phase extraction (SPE), dispersive solid phase extraction (dSPE), matrix solid-phase dispersion (MSPD), solid-phase micro-extraction (SPME) and stir bar sorptive extraction (SBSE) appear to

be appropriate and they have been applied for analysis of triazines in different kinds of vegetation samples [20, 21].

Matrix solid-phase dispersion is a sorptive extraction technique which involves the dispersion of the sample in a solid sorbent and subsequent elution with a relatively low solvent volume, allowing the simultaneous extraction and clean-up of analytes from solid samples [22]. If an additional cleanup step is necessary, it is possible to use the MSPD column with another sorbent at its bottom. This technique shows a high flexibility and selectivity due to the variety of possible combinations of both sorbents and elution solvents [23]. Due to its simplicity and high throughput, MSPD methods have been developed for the extraction of different pesticides residues from different plants and plant materials [24]; however references for the determination of triazines by MSPD are still scarce and furthermore in most cases few triazines are included in these studies [25-28].

The aim of this work was the development and validation of an effective and simple method based on Matrix Solid Phase Dispersion (MSPD) and Solid Phase Extraction (SPE) Clean-up followed by High Performance Liquid Chromatography (HPLC) coupled to Diode array Detection (DAD) for the simultaneous determination of nine triazine herbicides in seaweeds in order to be able of quantitate residues of these compounds in the range of the European maximum residue levels. Samples of three edible seaweeds were selected to illustrate the applicability of the method. To the best of our knowledge, no studies using MSPD have been done to extract these chemicals residues from seaweeds.

2. Experimental

2.1. Samples

Dried edible seaweeds: Sea Lettuce (*Ulva Lactuca*), Wakame (*Undaria pinnatifida*), and Nori (*Porphyra umbilicalis*), coming from aquaculture production, were purchased from a local market in A Coruña city, NW Spain. Samples were homogenized grounding them to a fine powder by an electric mill and stored in glass bottles out of light exposure until analysis.

2.2. Chemicals

(a) *Herbicide standards*- Herbicides (ametryn, atrazine, cyanazine, prometryn, propazine, simazine, simetryn, terbuthylazine and terbutryn) analytical standards were supplied by Sigma-Aldrich (Inc. St. Louis, MO, USA). The individual stock standard solutions of 1000 mgL^{-1} were prepared in methanol by exact weighing of high-purity substances and stored at -18ºC in the dark. Then a mixture of all the compounds was prepared in methanol containing 10 mg L^{-1} each individual triazine and stored at -18ºC.

All working solutions were daily prepared by appropriate dilution of the 10 mg L^{-1} standard solutions with methanol:water (1:1).

(b) *Solvents* – *n*-hexane 95% and methanol were superpurity Solvents from Romil (Cambridge, UK). Acetonitrile (HPLC grade) and ethyl acetate (PAR, solvents for analysis of pesticide residues by GC) for instrumental analysis were from Panreac (Barcelona, Spain). Milli-Q water was obtained from a purification system from Millipore (Billerica, MA).

(c) *Sorbents* - LC-8 Bulk packing and Supelclean™ ENVI-Carb II/PSA SPE Tube 6 ml (500 mg/500 mg) were from Sigma-Aldrich (Inc. St Louis, MO, USA).

(d) *Filters* - Polytetrafluoroethylene (PTFE) filters of 0.45µm were from Lida Manufacturing (Kenosha, WI, USA).

2.3. Materials and apparatus

A Visiprep® vacuum distribution manifold from Supelco (Bellefonte, PA, USA) was employed in the purification step. A Büchi R-3000 rotary evaporator (Büchi Labortechnic AG, Flawil, Switzerland) was used in the evaporation step.

Chromatographic analyses were carried out in a high performance liquid chromatography-diode array detector (HPLC-DAD). The system consisted of a 2695 pump with a 996 Diode Array Detector from Waters (Milford, MA, USA). The column was a stainless steel column (150 mm x 4.6 mm ID, particle size 5 µm) packed with Hypersil GOLD C_{18} chemical bonded phase from Thermo Scientific (Austin, TX, USA).

2.4. Extraction procedure

The optimization study was carried out using a pesticide free Sea Lettuce seaweed sample spiked at the 1 mg kg^{-1} level. 1.0000 g of dried seaweed sample was homogenised with 2.00 g of LC-8 in a glass mortar with a pestle for 5 min. The final mixture was transferred into a 12-mL SPE cartridge containing a dual sorbent layer of 1g Supelclean™ ENVI-Carb II/PSA (500/500mg). Once packed, MSPD/SPE columns were connected to a Visiprep® vacuum distribution manifold and washed with 10 mL of hexane. Elution was performed with 20 mL of ethyl acetate and 5 mL of acetonitrile (80:20) and the obtained eluate was evaporated to a drop in rotary-evaporator and brought to dryness by a gentle nitrogen stream. The residue was reconstituted in 1 mL methanol:water (1:1) and the solution was filtered through a 0.45 µm PTFE syringe filter.

2.5. HPLC-DAD conditions

The chromatographic analysis was carried out using the following ACN:H_2O gradient elution: ACN initial percentage of 30% (8 min), increased linearly to 40% in 5 min;

increased to 50% in 5 min, after which the percentage was returned to the initial conditions in 9 min. A constant mobile phase flow rate of 1 mL min^{-1} and 20 µL of sample volume were used.

The absorbance was measured continuously in the 200-400 nm range and peaks areas quantification were carried out at 222.7 nm in order to achieve maximum sensitivity. All triazine herbicides were identified initially by retention time and then by applying spectral contrast techniques (incorporated in Millenium[32] software) the homogeneity of the spectral peak was confirmed. Finally a spectral identification was carried out contrasting the spectrum with a standard library created in wavelength interval of 200-400nm.

3. Results and discussion

3.1. MSPD optimisation

In MSPD the analyte interacts with the solid support, the bonded phase and the dispersed matrix. For blending the sample, a glass mortar was used in order to avoid analyte losses that could occur with the use of materials such as porcelain [23]. In this technique the polarity of the sorbent and elution solvent are the key factors to achieve effectiveness of the extraction and purity of the final extract. Most methods reported have used reversed-phase materials bonded silica as the solid support. Theoretically, silica particles facilitate disruption of biological samples whereas the bonded alkyl chains contribute to dispersion and retention of lipophilic compounds [29]. C_8 and C_{18} are by far the most popular sorbents for analyte extraction from plant tissues; in this study C_8 was chosen as dispersant because it has been observed that C_8-bonded silica provided a more selective extraction with less co-elution compounds [30].

Although in some cases the MSPD extracts are clean enough to be directly subjected to instrumental analysis, a clean up step is often desirable. For this purpose an on-line clean-up step can be integrated into the sample preparation process by placing a layer of co-sorbent, obviously with different sorption behaviour, at the bottom of MSPD cartridge. Based on our experience on clean up of animal feed extracts [31], solid phase extraction was carried out employing a dual-layer tube containing carbon/primary secondary amine (ENVI^{TM}-Carb II/PSA) sorbents separated by PE frit. ENVI-Carb II is a graphitized non porous carbon that has a strong affinity towards planar molecules and can remove pigments (e.g., chlorophyll and carotenoids), and sterols. PSA is a polymerically bonded ethyklenediamine-N-propyl phase that contains both primary and secondary amines, that retains fatty acids, organic acids, sugars and some polar pigments [32].

Selection of elution solvent is function of analyte polarity, since the target analytes should be efficiently desorbed while the remaining components should be retained in the column. The generally employed solvents in the literature for elution of triazines in vegetable extracts from MSPD-SPE systems are acetonitrile [25], dichloromethane [27] and specially ethyl acetate [26,28,33]. In this study ethyl acetate and acetonitrile were tested; dichloromethane was avoided, according to principles of green chemistry, for being a chlorinated solvent.

The optimisation of the MSPD-SPE procedure was carried out by spiking the sorbents with 1 mL of a standard solution containing 1 mg L^{-1} of each triazine. As ethyl acetate is the most common solvent for elution of triazines in MSPD procedures and the time consumed in the evaporation step is much lower with ethylacetate than with acetonitrile, preliminary assays were carried out with ethyl acetate. In order to determine solvent volume required for the complete elution of the target analytes, eluates of 5 mL were collected and the obtained results showed that at least 15 mL of ethyl acetate were necessary for recovering all compounds from the MSPD-SPE system. Recoveries obtained were higher than 90% for all compounds except for symetrin (70%), which although it has a lower value than for the rest of triazines, it is acceptable in trace analysis. However, with a view to improving the recovery of symetrin, the sequence of 10 mL of ethyl acetate and 5 mL of acetonitrile was assayed and recoveries obtained were similar than those achieved with ethyl acetate for most of compounds but recovery of simetryn increased until 86%. Therefore the combination 10 mL ethyl acetate + 5 mL acetonitrile was selected as elution solvent.

The selected conditions were applied to samples of sea lettuce, which did not contain triazine residues at detectable concentrations. For this purpose, 1.0000 g of dried seaweed was spiked at the 1 mg kg^{-1} level (equivalent to 0.08 mg kg^{-1} of fresh tissue) and subjected to the procedure previously optimized with standards. Recoveries obtained for spiked seaweed were between 50-65% for all analytes which implies a strong interaction of all studied compounds with the matrix. Therefore additional volumes of ethyl acetate and/or acetonitrile were collected but very unsatisfactory recoveries were obtained in all cases, and only the tandem of 20 mL ethyl acetate and 5 mL acetonitrile achieved recoveries higher than 60% for all compounds. Several authors have pointed out that washing the column with a solvent, such as hexane or water, prior to elution of the target analytes can have a huge influence on the performance of the MSPD method [29,30]. Therefore an experiment consisted on rinsing the sample with 10 mL of hexane, prior to analytes elution with 20 mL ethyl acetate and 5 mL acetonitrile, was carried out. With this procedure, schematized in Figure 1, satisfactory recoveries were obtained for all triazines (up to 80%). As an illustration of the results

obtained, Figure 2 shows the chromatograms corresponding to unspiked and spiked sea lettuce extracted and purified under the procedure considered.

3.2. Method validation

The method was validated in terms of linearity, recoveries, precision and limits of detection and quantification. The validation data are presented in Table 1. All quantitative results were calculated using matrix matched standards prepared by spiking the final extracts from un-spiked samples of sea lettuce with different levels of triazines as recommended by the European guidelines [34]. Good linearity of the calibration curves were obtained for all compounds over the whole range (0.1-2 mg kg^{-1} dried sample) evaluated by duplicate analysis at five different concentration levels. The target compounds showed coefficients of determination (R^2) higher than 0.9992 for all triazines except for prometrin and terbutryn which showed only slightly lower R^2 (> 0.995).

The matrix effect was evaluated since signal suppression or enhancement can severely compromise quantitative analysis of the compounds at trace levels, and therefore can greatly affect the method reproducibility and accuracy. The matrix effect was studied by comparison of the slopes of the calibration curves in solvent and in the extract obtained after the MSPD procedure. Both the solvent and the matrix calibration curves had good linearity, with determination coefficients higher than 0.9999 for solvent calibration curves and 0.9952 for matrix matched ones. All compounds showed a strong matrix effect since the deviation of the matrix calibration slope from the solvent calibration slope was higher than 20% (between 29-42%), which indicated the need of using matrix matched standards for quantification purposes.

The accuracy and precision of the proposed method was investigated by analysis of five replicates of uncontaminated sea lettuce samples spiked at two different concentration levels (1 and 0.1 mg kg^{-1} dried sample, equivalent to 0.08 and 0.008 mg kg^{-1} fresh sample respectively). The lowest spiked concentration was selected to test the method performance at the recent restrictive residue level set by European Union Legislation for simazine in edible seaweeds [35]. The obtained results demonstrated that the method achieved satisfactory recoveries in the range of 75-100% in all cases, with associate standard deviations below 7% for all compounds, which are in the acceptance range [34]. The obtained recoveries and relative standard deviation are comparable or even better to those provided by other authors for the determination of some of these pollutants by MSPD in fruits and vegetables [27, 28, 33].

The limits of detection (LODs) and limits of quantification (LOQs) were calculated as the minimum amount of target analyte that led to a chromatogram peak with a signal-to-noise ratio of 3 and 10 respectively, determined experimentally from fortified samples

[34]. As it can be seen in Table 1, the LODs varied from 1.4 to 3.8 µg kg^{-1} and LOQs values from 4.1-7.3 µg kg^{-1} in fresh sample. Therefore, the obtained LODs and LOQs were satisfactory and allow the determination of these compounds at the levels required by the legislation of seaweed for human consumption. By comparing the LODs obtained by the proposed methodology with those reported in the literature using MSPD in horticultural matrices, better sensitivity is attained [27, 28, 33].

Reliability of the method was evaluated in terms of recovery by spiking two edible seaweed samples: a red one (nori) and a brown one (wakame) at a concentration level of 1 and 0.1 mg kg^{-1} dried sample. The analytical recoveries, calculated using matrix matched standards, obtained for five replicates (n=5) of the samples spiked with the triazinic herbicides are presented in Table 2. As it can be seen the recovery values obtained for wakame ranged between 85 and 100% for all compounds, except for prometrin at the low level (120%), with RSD lower than 7%. In the case of nori recoveries fluctuated from 85-90% with RSD values below 2% at the high level whereas they were between 65-85% with RSD below 9% at the low level. Therefore we can conclude that this method could be established as a suitable method to routine analysis to screen trace levels in different types of seaweed in compliance with EU directives.

4. Conclusions

A procedure for the analysis of nine triazines from seaweed samples based on MSPD and SPE has been developed. The method uses C_8 as dispersant with ENVI-Carb™/PSA co-column and *n*-hexane as washing solvent followed by a combination of 20 mL ethyl acetate and 5 mL acetonitrile as elution solvent. The developed method provides satisfactory accuracy and precision for the determination of triazines in seaweed with LODs and LOQs adequate to carry out analysis of samples in the concentrations required by the European Union regulations. The method was successfully applied to the analysis of three seaweed samples (sea lettuce, wakame and nori). The main advantages of this methodology when compared with conventional methods of sample preparation to screen triazines in vegetable matrices are easy of work-up, fast, low cost, avoidance of clean-up procedure, as well as the significant reduction of organic solvents in agreement with the principles of the Green Chemistry.

Acknowledgements

The authors acknowledge the financial support of the “Ministerio de Educación y Ciencia” (Project CTQ2007-63949/BQU) and the “Xunta de Galicia” (Projects 09MDS038103PR and 2010/52). N. Rodríguez-González also thanks the “Xunta de Galicia” for the concession of her award under the program “Campus del Mar”.

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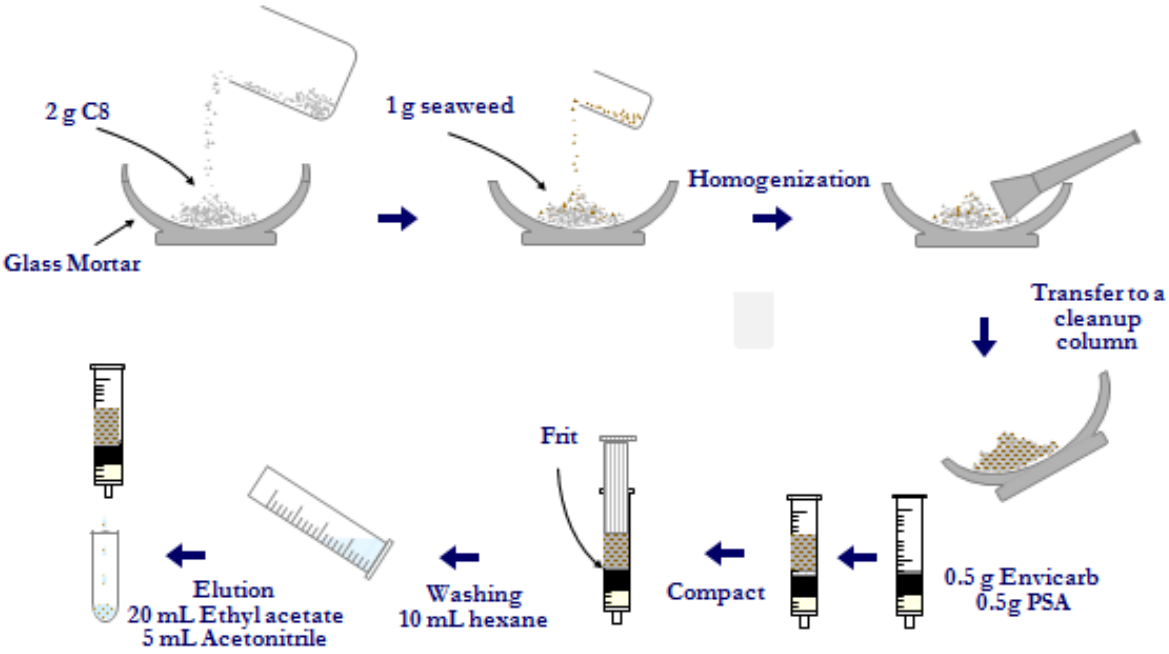

Figure 1. Scheme of the final conditions of MSPD-SPE procedure

(a)

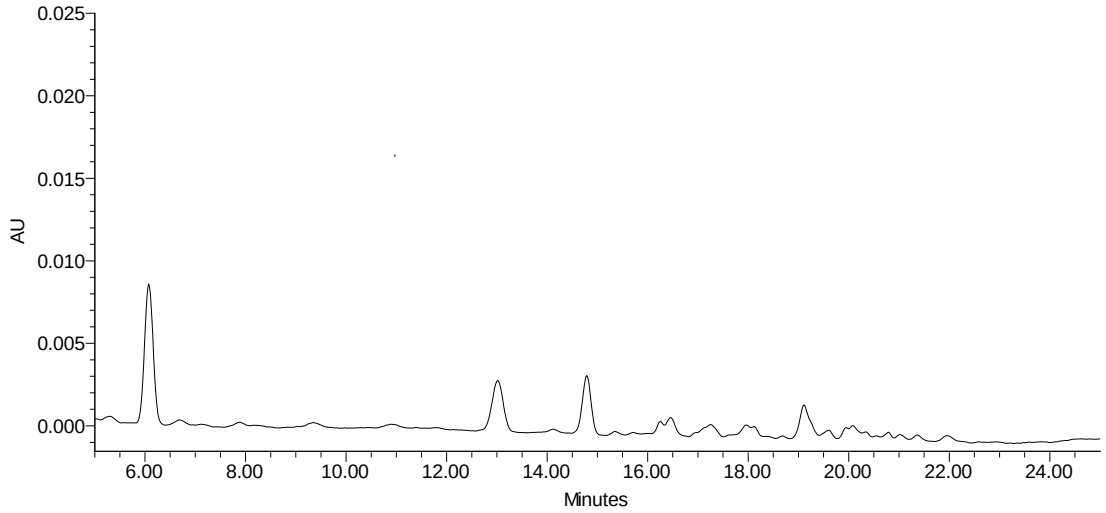

Figure 2. (a) MSPD sea lettuce extract chromatogram. Target compounds are numbered as follows: (1) simazine, (2) cyanazine, (3) simetryn, (4) atrazine, (5) ametryn, (6) propazine, (7) terbuthylazine, (8) prometryn, (9) terbutryn

(b)

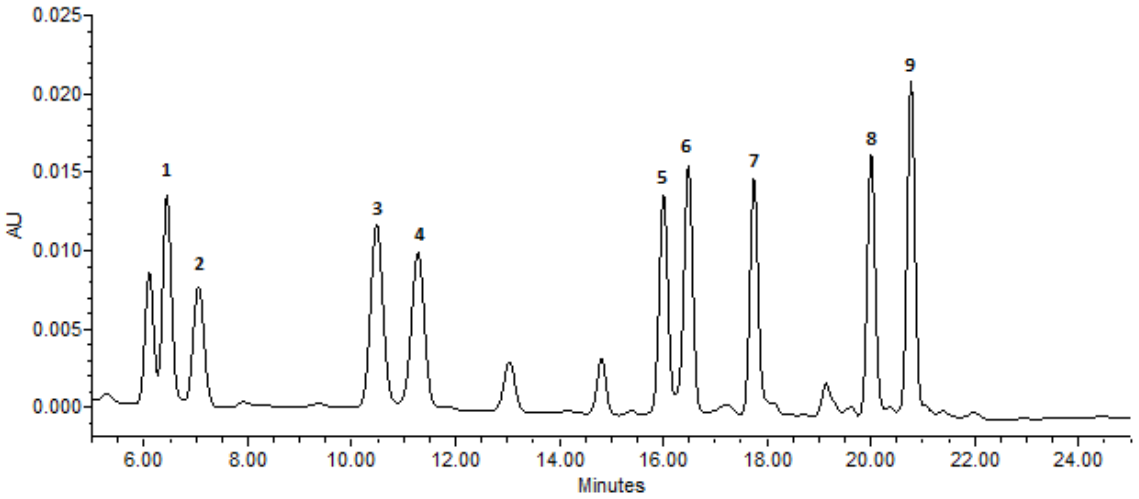

Figure 2. (b) MSPD spiked sea lettuce extract chromatogram. Target compounds are numbered as follows: (1) simazine, (2) cyanazine, (3) simetryn, (4) atrazine, (5) ametryn, (6) propazine, (7) terbuthylazine, (8) prometryn, (9) terbutryn

Table 1. Validation data for the MSPD-HPLC-DAD method obtained employing sea lettuce seaweed

Compound	LOD* (µg kg^{-1})	LOQ* (µg kg^{-1})	Correlation coefficient (R^2)	Recovery ± RSD (%) (n=5)	
				0.1 mg kg^{-1}	1 mg kg^{-1}
Simazine	3.6	6.5	0.9997	81.2±6.3	88.2±0.9
Cyanazine	3.0	7.3	0.9998	80.2±5.7	79.5±0.6
Simetryn	3.8	6.2	0.9999	80.8±5.3	83.0±2.5
Atrazine	2.7	5.3	0.9992	85.9±5.5	81.0±2.8
Ametryn	2.9	5.9	0.9997	90.8±7.3	83.0±1.0
Propazine	3.6	6.0	0.9992	91.7±5.1	91.8±0.6
Terbuthylazine	1.5	4.3	0.9996	76.0±7.4	84.8±1.7
Prometryn	1.4	4.1	0.9973	82.8±7.2	96.6±2.8
Terbutryn	1.6	4.5	0.9952	86.1±3.6	83.2±1.1

*Results expressed in µg kg^{-1} fresh sample

Table 2. Mean recoveries and RSD values of triazines in wakame and nori samples (n=5) spiked at two levels applying MSPD-HPLC- DAD method

Compound	Wakame		Nori	
	Recovery ± RSD (%) (n=5)			
	0.1 mg kg^{-1}	1 mg kg^{-1}	0.1 mg kg^{-1}	1 mg kg^{-1}
Simazine	98.7±4.7	90.4±4.1	83.6±6.7	85.9±1.5
Cyanazine	87.0±3.4	85.7±1.2	64.8±8.8	87.5±1.9
Simetryn	94.2±4.5	84.6±0.3	65.1±3.0	85.5±1.4
Atrazine	92.3±0.5	89.1±1.4	78.6±1.5	89.1±1.7
Ametryn	102.1±3.9	89.6±1.2	77.5±2.2	91.0±0.8
Propazine	92.9±6.8	90.5±1.5	64.6±3.3	90.5±1.2
Terbuthylazine	86.0±1.0	90.8±1.4	70.4±2.4	86.7±1.4
Prometryn	120.2±3.1	91.0±1.8	70.9±2.8	86.8±0.7
Terbutryn	99.1±0.7	89.4±1.8	64.7±5.2	85.4±1.0

DEVELOPMENT OF A MATRIX SOLID PHASE DISPERSION METHODOLOGY FOR THE DETERMINATION OF TRIAZINE HERBICIDES IN MUSSELS

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ABSTRACT

A method based on Matrix Solid Phase Dispersion (MSPD) for determination of nine triazines in mussels has been optimized in terms of the sorbents used for extracting and cleaning-up. Two dispersing agents: C_{18} and florisil, and eight cleanup co-sorbents: florisil, silica, silica/alumina, Envi^{TM}Carb, Envi-Carb-II/PSA, SAX/PSA, Envi-Carb-II /SAX/PSA and C_{18} were assayed. Analytes were eluted using 20 mL of ethyl acetate and 5 mL of acetonitrile and finally the extract was concentrated to dryness, re-constituted with 1 mL methanol and determined by HPLC-DAD. The best results were obtained with C_{18} as dispersing agent and Envi-Carb-II/SAX/PSA as clean-up co-column. Recoveries ranged between 79 and 99 % and repeatability and reproducibility were below than 16 % for all compounds. The linearity of the calibration curves yielded the $R^2 \geq 0.9993$. The LOQ values ranged from 0.10 to 0.18 mg kg^{-1} dried sample. Finally the method was applied to the analysis of mussel samples from Galicia (NW Spain).

Keywords: Triazines. Mussel. Matrix solid phase dispersion. High performance liquid chromatography. Diode array detection.

1. Introduction

The accumulation of environmental chemical contaminants in fish and shellfish pose a potential human health hazard. Coastal areas are subject to strong anthropogenic pressures as a consequence of their location between land and sea and they also receive fluxes of pesticides mainly of agricultural origin (Carafa et al., 2007, Fernández, Fernández-Boán, Verísimo, & Freire, 2013). Impacts of herbicides in the aquatic ecosystem are both direct and indirect, spanning from instantaneous effects to long-term effects as bioaccumulation and biomagnification through the food chain (Carafa et al., 2007).

Triazines are a group of herbicides that are present in the ten most-used herbicide formulations in Europe. Triazines have been used extensively as herbicides to provide pre- and post-emergence of grasses, crops and many weeds in cereals (Wang et al.,

2010). Because of their relatively low soil adsorption and high solubility in water, these compounds migrate from soil to water (Andreu, & Picó, 2004) and due to the water cycle, fluxes of them reach seawater and affect the marine biota, having the coastal systems little capacity to degrade these compounds (Fernández et al., 2013). Therefore, they are considered as an important class of chemical pollutants and atrazine and simazine have been included in the Endocrine Disruption Screening Program by the U.S. Environmental Protection Agency (2009).

As a result, the European Union has also included simazine and atrazine in the list of 33 priority substances in the EU Water Framework Directive (2000/60/EC) by way of Decision 2455/2001/EC. Recently, terbutryn has been added to the list of priority substances by way Commission Proposal for a Directive (Proposal COM (2011)876). Moreover, the Directive 2008/105/EC sets the Environmental Quality Standards (EQS) for these compounds in water and also shows the need to set EQS for these compounds in sediments and/or biota. Although limit values have not established yet by European Union for triazines in fish and fishery products, the U.S. Food and Drug Administration (FDA) has set a tolerance level of 12 mg kg^{-1} ww for simazine in fish (2011).

Commission Proposal for a Directive COM2011/876/EC, 2008/105/EC as regards priority substances in the Field of Water Policy. COD 2011/0429, and 31 January (2012

Aquatic organisms, such as fish and shellfish, are able to accumulate pesticide residues in much higher concentration than the surrounding water (LeDoux, 2011). Thus, monitoring their content in seafood is necessary to estimate human exposure to these substances. Bivalves are suitable organisms for using in environmental monitoring programs due to their wide geographical distribution, great sensitivity to environmental pollutants and high accumulation rate; furthermore they are stationary and are normally the dominant species in their habitat (Binelli, Ricciardi, Riva, & Provini, 2006).

The more frequently used methodologies for the analysis of triazines in samples of animal origin employ solvent extraction procedures such as soxhlet (Rosenblum, Hieber, & Morgan, 2001), shake flask (Baranowska, Barchanska, Abuknesha, Price, & Stalmach, 2008; Tsuda, Nakamura, Inoue, & Tanaka, 2009), sonication (Carafa et al., 2007; Salvadó, Quintana, & Hidalgo, 2006), microwave-assisted extraction (MAE) (Cheng et al., 2007; Fernández, et al., 2013) and pressurized liquid extraction (PLE) (Damásio et al., 2010; Köck et al., 2010). Nevertheless, they generally need to add a clean-up step to decrease the presence of interferents in the final extract to reduce the detection limits of the methods and to avoid inaccurate results in the chromatographic determination. In the last years, different innovation procedures have been developed and applied for the determination of pollutants in complex matrices with improved capabilities, reduced clean up and concentration steps, the avoidance of toxic solvents

and improved limits of detection. In this context, modern extraction techniques such as dispersive solid phase extraction (dSPE), matrix solid-phase dispersion (MSPD), solid-phase micro-extraction (SPME) and stir bar sorptive extraction (SBSE) appear to be appropriate (Beceiro-González, González-Castro, Muniategui-Lorenzo, López-Mahía, P., & Prada-Rodríguez, 2012). The main difference among these techniques is that the retention in the two latter techniques is based on the distribution equilibrium between the sample matrix and a non-miscible sorbent phase.

MSPD is an extraction procedure which combines aspects of several analytical techniques allowing sample homogenization, disruption, extraction, fractionation and clean-up within a single process (Barker, 2000a). In MSDP the solid sample is blended in a mortar with an appropriate sorbent to obtain complete disruption and dispersion of the sample on the solid support. The blend is packed into a column from which the analytes are eluted with a relatively low solvent volume (Ramos, González, & Ramos, 2009). Often, a co-sorbent material is placed at the bottom of the column to be filled with the blended sample to assist the extract clean up (Capriotti, Cavaliere, Laganá, Piovesana, & Samperi, 2013).

The key factors for the success of MSPD are its feasibility, flexibility, versatility, high throughput, low cost and rapidity. MSPD methods have been developed for the extraction of triazine residues from different plants and plant materials (Ramos et al., 2009; Rodríguez-González et al., 2014; Wen et al., 2012); however references for the determination of triazines in animal tissues by MSPD are scarce. As far as we know, there are only two references in the literature devoted to the determination of triazines in fish by MSPD and furthermore few triazines are included in these studies (Gaunt & Barker, 2000; Souza-Caldas et al., 2013).

The aim of this work was the development and validation of an effective, simple and fast method for the simultaneous determination of nine triazine herbicides in mussels based on Matrix Solid Phase Dispersion (MSPD) and Solid Phase Extraction (SPE) clean-up followed by High Performance Liquid Chromatography (HPLC) coupled to Diode array Detection (DAD). To the best of our knowledge, no studies using MSPD have been done to extract these chemicals residues from shellfish. As an application, the analysis of mussel samples from Galicia (NW Spain) was carried out.

Galicia is the highest producer of mussels in Europe and the fish and shellfish industry is very important in this area, exporting their products around the world. Therefore the study of levels of these contaminants on fish and fishery products is of a great economic and environmental importance for Galicia.

2. Material and methods

2.1. Samples

Mussels (*Mytillus galloprovincialis*) were purchased from a local market in A Coruña city (Northwest of Spain). Mussels were removed from the shell, homogenized and freeze-dried. Then samples were homogenized again grounding it to a fine powder by an electrical mill and finally they were stored in glass bottles out of light exposure until analysis.

2.2. Chemicals

(a) *Herbicide standards*- Herbicides (ametryn, atrazine, cyanazine, prometryn, propazine, simazine, simetryn, terbuthylazine and terbutryn) analytical standards were supplied by Sigma-Aldrich (Inc. St. Louis, MO, USA). The individual stock standard solutions of 1000 mg L^{-1} were prepared in methanol by exact weighing of high-purity substances and stored at -18º C in the dark. Then a mixture of all the compounds was prepared in methanol containing 10 mg L^{-1} each individual triazine and stored at -18º C. All working solutions were daily prepared by appropriate dilution of the 10 mg L^{-1} standard solutions with methanol.

(b) *Solvents – n*-hexane 95 % and methanol were superpurity Solvents from Romil (Cambridge, UK). Acetonitrile (ACN) (HPLC grade) and ethyl acetate (PAR, solvents for analysis of pesticide residues by GC) for instrumental analysis were from Panreac (Barcelona, Spain). Milli-Q water was obtained from a purification system from Millipore (Billerica, MA).

(c) *Sorbents – Bulk packing:* Envi-18 and LC-Florisil were from Sigma-Aldrich (Inc. St Louis, MO, USA). *SPE tubes*: Envi-Florisil (1 g and 2 g), LC-Silica (1 g), LC-Silica/Alumina (1 g/1 g), Envi^{TM} Carb (1 g), Envi-Carb-II/PSA (500 mg/500 mg), SAX/PSA (500 mg/500 mg), Envi-Carb II/SAX/PSA SPE (500 mg/500 mg/500 mg) were from Sigma-Aldrich (Inc. St Louis, MO, USA); C18 SepPak Plus was from Waters (Milford, MA, USA).

(d) *Filters* - Polytetrafluoroethylene (PTFE) filters of 0.45 μm were from Teknocroma (Barcelona, Spain).

2.3. Materials and apparatus

A Visiprep® vacuum distribution manifold from Supelco (Bellefonte, PA, USA) was employed in the purification step. A Büchi R-3000 rotary evaporator (Büchi Labortechnic AG, Flawil, Switzerland) was used in the evaporation step.

Chromatographic analyses were carried out in a high performance liquid chromatography-diode array detector (HPLC-DAD). The system consisted of a 2695 pump with a 996 Diode Array Detector from Waters (Milford, MA, USA). The column

was a stainless steel column (150 mm x 4.6 mm ID, particle size 5 µm) packed with Hypersil GOLD C_{18} chemical bonded phase from Thermo Scientific (Austin, TX, USA).

2.4. Extraction procedure

Optimization experiments of the several sorbents tested were carried out by analysing a triazine-free freeze-dried mussel sample spiked with the triazines to obtain a final concentration of each analyte in the mussel of 2 mg kg^{-1}. Under final working conditions, 0.5 g of freeze-dried mussel sample was homogenised with 2 g of Envi-C18 in a glass mortar with a pestle for 5 min. The final mixture was transferred into a 20-mL SPE cartridge containing a triple sorbent layer of 1.5 g Supelclean™ Envi-Carb-II/SAX/PSA (500/500/500 mg). Once packed, MSPD/SPE columns were connected to a Visiprep® vacuum distribution manifold and washed with 10 mL of hexane. Elution was performed with 20 mL of ethyl acetate and 5 mL of ACN and the obtained eluate was evaporated to a drop in rotary-evaporator and got to dryness by a gentle nitrogen stream. The residue was reconstituted in 1 mL methanol and the solution was filtered through of a 0.45 µm syringe filter of PTFE.

2.5. HPLC-DAD conditions

The chromatographic analysis was carried out using the following ACN:H_2O gradient elution: ACN initial percentage of 30 % (8 min), increased linearly to 40 % in 5 min; increased to 50 % in 5 min, after which the percentage was returned to the initial conditions in 9 min. A constant mobile phase flow rate of 1 mL min^{-1} and 20 µL of sample volume were used. The absorbance was measured continuously in the 200-400 nm range and peaks areas quantification were carried out at 222.7 nm in order to achieve maximum sensitivity. All triazine herbicides were identified initially by retention time and then by spectral identification contrasting the spectrum with a standard library created in the same wavelength interval.

3. Results and discussion

3.1. MSPD optimisation

The effectiveness of an MSPD procedure depends on the sorbent/solvent combination. The following parameters were taking into consideration in the univariate optimization of the MSPD procedure: type and amount of dispersing agent and sorbents used for the clean-up. Most methods reported have used reversed-phase materials, such as C8 and C18-bonded silica as the solid support, whereas normal phase such as florisil or silica are used less frequently (Kristenson, Ramos, & Brinkman, 2006). The lipophilic character of the reversed phase material is believed to facilitate disruption, dispersion and retention of lipophilic compounds whereas normal phase materials interact with

sample components only by adsorption and, obviously, are not able to dissolve the sample matrix (García-López, Canosa, & Rodríguez, 2008). Although in some cases the MSPD extracts are clean enough to be directly subjected to instrumental analysis, a clean-up step is often desirable. For this purpose an on-line clean-up step can be integrated into the sample preparation process by placing a layer of co-sorbent with different sorption behaviour, at the bottom of MSPD cartridge.

For optimization of the MSPD procedure 0.5 g of pesticide free mussel sample was spiked at the 2 mg kg^{-1} level. A reversed phase (C18) and a normal phase (florisil) sorbents were tested as dispersants (1.5 g each). C18 was chosen because it is by far the most popular sorbent, especially for analyte extraction from animal tissues (Kristenson et al., 2006) because cell membranes could be disrupted through the solubilisation of phospholipid components in C_{18} material. Florisil was selected because it has been reported as the more efficient normal phase material in terms of lipid retention from fish and shellfish tissues (Negreira, Rodríguez, Rodil, Rubí & Cela, 2013).

First assays were carried out with florisil because the combination of normal phase as dispersant and reversed phase as clean up co-sorbent has been reported as more efficient in terms of lipids retention in fish (Canosa, Rodríguez, Rubí, Ramil & Cela, 2008). For this purpose, florisil (1.5 g) as dispersant was assayed with Envi-Carb (1 g) and C18 (1 g) as co-sorbents. Based on our experience, elution of the cartridges was performed with 20 mL of ethyl acetate and 5 mL of acetonitrile after rinsing with 10 mL of hexane (Rodríguez-González et al., 2014). The extracts obtained using C18 were very dark, indicating coelution of matrix interferences and were not analysed. Envi-carb provided extracts clean enough to be injected, however recoveries obtained ranged between 29 and 69 %, and therefore the use of florisil as dispersant was discarded.

Considering C18 (1.5 g) as dispersant the following co-sorbents were assayed: florisil (1 g and 2 g), silica (1 g), silica and alumina (1 g/1 g), Envi-Carb-II/PSA (0.5 g/0.5 g), SAX/PSA (0.5 g/0.5 g), and Envi-Carb-II/SAX/PSA (0.5 g/0.5 g/0. 5g). Experiments carried out using florisil (1 g) and silica (1 g) provided extracts very dark and with solid particles and were not analysed further. The use of SAX/PSA (0.5 g/0.5 g), and especially silica/alumina (1 g/1 g) and florisil (2 g) led to intensely coloured extracts; however they could be processed because the filtration through PTFE filters considerably decreased the colour of the extracts. Envi-Carb-II/PSA (0.5 g/0.5 g) and Envi-Carb-II/SAX/PSA (0.5 g/0.5 g/0.5 g) provided extracts clean enough before filtration through PTFE filters, which indicates the importance of using graphitized carbon black on the cleaning of mussel extracts.

Recoveries obtained with florisil and silica/alumina were lower than 80 % for all compounds, while the use of SAX/PSA yielded recoveries ranging from 72 to 89 %.

Satisfactory recoveries were achieved with systems based on carbon, Envi-Carb-II/PSA and Envi-Carb-II/SAX/PSA, with values ranging from 79 to 96 % for the first system and from 85 to 102 % for the second one (Figure 1). As well as recoveries, taking into account the higher deviation of the results in the case of Envi-Carb-II/PSA when comparing with Envi-Carb-II/SAX/PSA, the system Envi-Carb-II/SAX/PSA was chosen as clean up co-sorbent. ENVI-Carb-II is a graphitized non porous carbon that has a strong affinity towards planar molecules and can remove pigments (e.g., carotenoids), and sterols. In addition, PSA is a polymerically bonded ethylenediamine-N-propyl phase that contains both primary and secondary amines, that retains fatty acids, organic acids, sugars and some polar pigments. SAX is a quaternary amine that offers additional ion exchange capacity for removing matrix components providing low UV and MS extractables for lower background and greater sensitivity.

In developing the method, it was observed that after several injections ghost peaks appeared. After checking the instrument and restoring the column performance by running a strong solvent, the ghost peaks were attributable to a late eluting compound present in sample. Therefore the sample preparation method was checked, in terms of recovery, increasing the amount of dispersant to 2 g and the problem of ghost peaks was resolved. Thus, 2 g of C18 as dispersant and Envi-Carb-II/SAX/PSA (0.5 g/0.5 g/0.5 g) as clean-up co-sorbents were selected for the whole procedure. Figure 2 shows the chromatograms corresponding to unspiked and spiked mussel sample extracted and purified under the considered procedure.

3.2. Method validation

The method was validated in terms of linearity, accuracy, precision and limits of detection and quantitation according to "validation parameters and criteria" from SANCO Guidelines for method validation and quality control procedures for pesticide residue analysis in food and feed (Directorate of General Health and Consumer Protection. Document nº SANCO/12571, 2013). The validation data are presented in Tables 1 and 2. Quantitative results were calculated using matrix matched standards prepared by spiking the final extracts from blank samples of mussels with different levels of triazines. The linearity of the calibration curves was calculated at a concentration range between 0.2-4 mg kg^{-1} dried sample by duplicate analysis at six different concentration levels. As can be seen in Table 1 excellent linearities were obtained with coefficients of determination (R^2) higher than 0.9993 for all triazines.

The limits of detection (LODs) and quantitation (LOQs), calculated as the minimum amount of target analyte that led to a chromatogram peak with a signal-to-noise ratio of 3 and 10, respectively, are shown in Table 1. The LODs varied from 0.036 to 0.068 mg kg^{-1} and LOQs from 0.10 to 0.18 mg kg^{-1}, referred to the freeze-dried sample. The

reduction in the weight of mussel samples during freeze-drying accounted for ca. 80% (0.5 g of freeze-dried sample equivalent to ca. 2.5 g of fresh tissue); therefore, approximately 5-fold lower LOQ values are achieved when referred to fresh sample. By comparing the LOQs obtained by the proposed methodology with those reported in the literature using MSPD in fish, better sensitivity is attained (Gaunt & Barker, 2000; Souza-Caldas et al., 2013).

Precision and accuracy of the proposed method were determined by spiking 0.5 g of uncontaminated mussel sample at two concentration levels (0.2 y 2 mg kg^{-1}). The accuracy of the method was evaluated regarding the recovery assay by analysis of five replicates at each fortification level. The obtained results demonstrated that the method achieved satisfactory recoveries for all compounds in the range of 79-99 % for the low level and of 91-97 % for the high level (Table 2), which indicate that the method meets the requirements stipulated (DG SANCO/12571/2013). The precision of the method, expressed as the relative standard deviation (RSD), was evaluated measuring five replicates samples at the same day (intraday precision) and six replicate samples on three consecutive days (inter-day precision). RSD values lower than 10 % and 16 % for intraday and interday precision respectively were obtained (Table 2), which are in the acceptance range of the DG SANCO/12571/2013 of the European Quality Control Guidelines (RSD < 20%). It is worth noting that the obtained recoveries and relative standard deviation are comparable or even better to those provided by other authors for the determination of some of these pollutants by MSPD in fish (Gaunt & Barker, 2000; Souza-Caldas et al., 2013).

Finally, the developed method was applied to analyse the different target analytes in five mussel samples purchased from local markets of A Coruña (NW Spain). Although none of them contained detectable amount of target pesticides, studies regarding the presence of these compounds are required on shellfish harvested from aquaculture ponds because these areas are subject to shore side contaminant discharges.

4. Conclusions

The suitability of a procedure based on MSPD and SPE for the extraction of nine triazines from shellfish samples has been demonstrated for the first time. The method uses C18 as dispersant with ENVI-Carb-II/SAX/PSA co-column followed by a combination of 20 mL ethyl acetate and 5 mL acetonitrile as elution solvent. The developed method provides satisfactory accuracy and precision for the determination of triazines in mussels. The main advantages of this methodology when compared with classical methods of sample preparation to determine triazines in animal tissues are easy of work-up, fast, low cost, avoidance of clean-up procedure, as well as the significant

reduction of organic solvents and energy requirements in agreement with the principles of the Green Chemistry.

The proposed method was applied to the analysis of mussel samples from Galicia aquaculture ponds. Although the triazines under study were not detected in the samples, the analysis of these compounds in mussels is of great interest in order to evaluate risks for human health and also to control the quality of the marine environment.

Acknowledgements

This work has been supported by Program of Consolidation and Structuring of Units of Competitive Investigation of the University System of Galicia (Xunta de Galicia) potentially cofounded by European Regional Development Fund (ERDF) in the frame of the operative Program of Galicia 2007-2013 (reference: GRC2013-047). N. Rodríguez-González also thanks the "Xunta de Galicia" for the concession of her award under the program "Campus del Mar".

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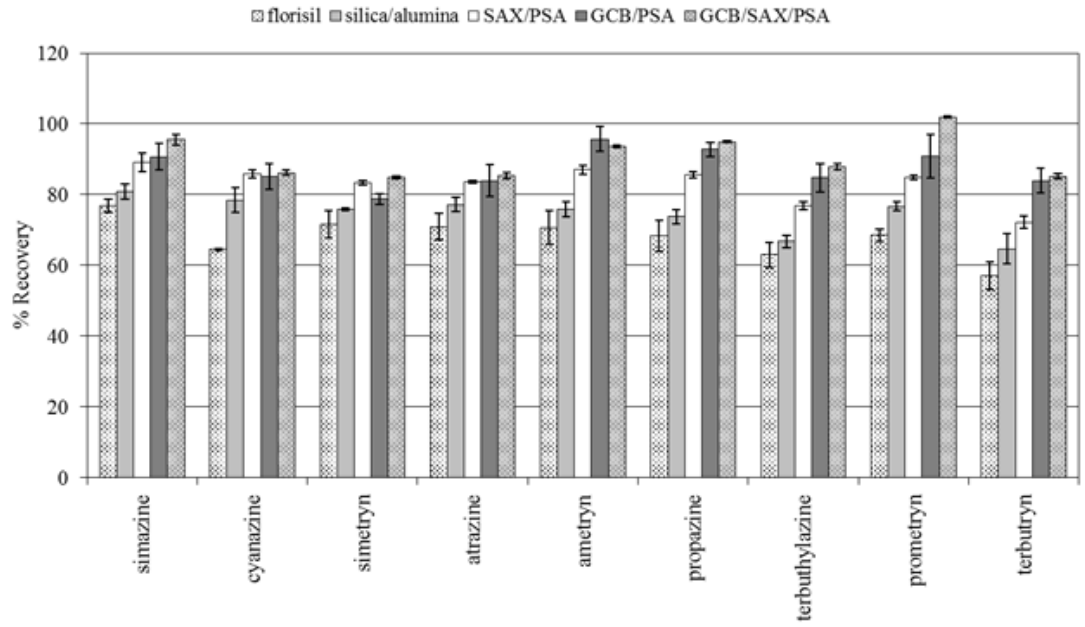

Figure 1. Analytical recoveries of triazines from mussel samples using C18 as dispersant with different cleanup co-sorbents

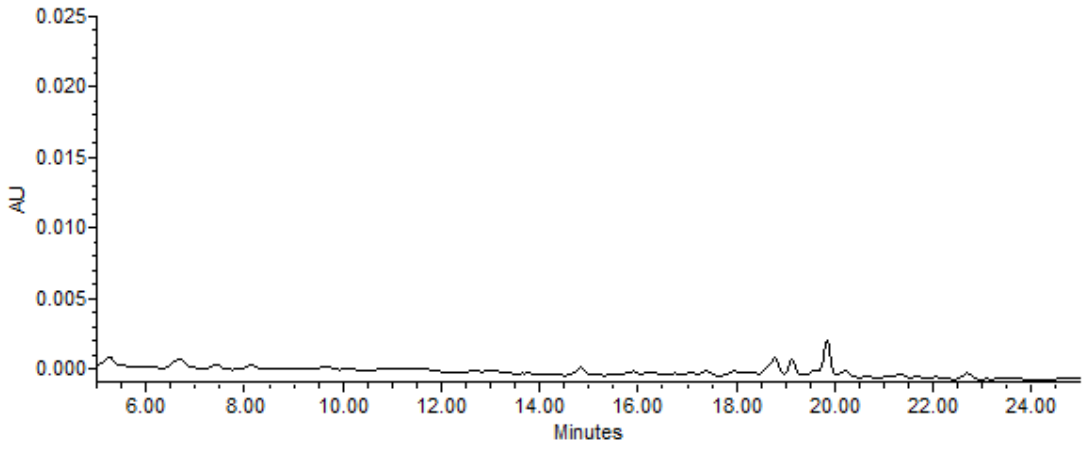

Figure 2. (a) MSPD mussel extract chromatogram. Target compounds are numbered as follows: (1) simazine, (2) cyanazine, (3) simetryn, (4) atrazine, (5) ametryn, (6) propazine, (7) terbuthylazine, (8) prometryn, (9) terbutryn

(b)

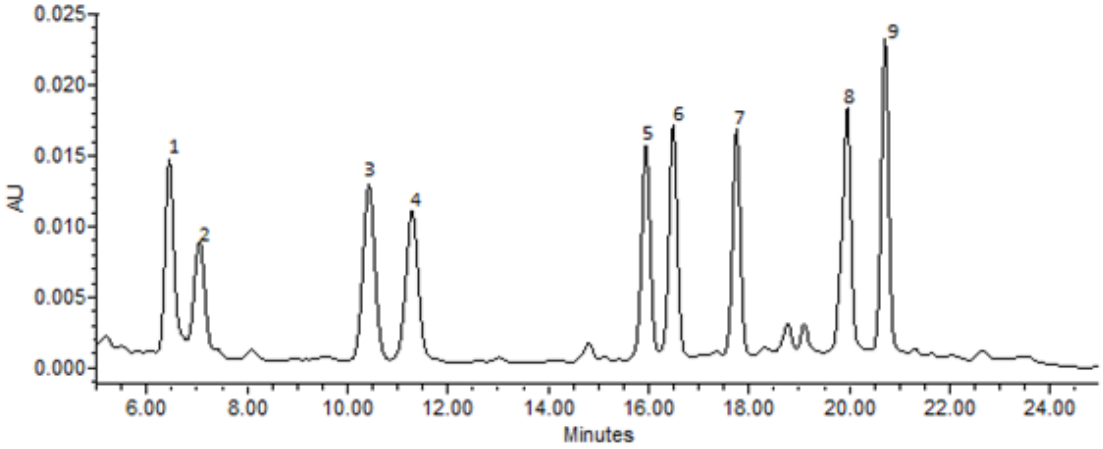

Figure 2. (b) MSPD spiked mussel extract chromatogram. Target compounds are numbered as follows: (1) simazine, (2) cyanazine, (3) simetryn, (4) atrazine, (5) ametryn, (6) propazine, (7) terbuthylazine, (8) prometryn, (9) terbutryn

Table 1. Coefficient determination (R^2) and LODs and LOQs

Compound	Determination coefficient (R^2)	LOD (mg kg^{-1})	LOQ (mg kg^{-1})
Simazine	0.9999	0.050	0.13
Cyanazine	0.9998	0.068	0.18
Simetryn	0.9993	0.038	0.10
Atrazine	0.9993	0.043	0.12
Ametryn	0.9996	0.045	0.12
Propazine	0.9993	0.041	0.11
Terbuthylazine	0.9996	0.046	0.12
Prometryn	0.9993	0.049	0.13
Terbutryn	0.9997	0.036	0.10

Table 2. Precision and accuracy of target compounds in mussel samples with MSDP-HPLC-DAD

Compound	Repeteability (%RSD, n=5)		Reproducibility (%RSD, n=6)		Accuracy (%) Recovery (n=5)	
	0.2 mg kg	2 mg kg^{-1}	0.2 mg kg^{-1}	2 mg kg^{-1}	0.2 mg kg^{-1}	2 mg kg^{-1}
Simazine	1.5	1.6	7.5	2.8	99	97
Cyanazine	10	1.9	12	2.3	96	95
Simetryn	3.6	2.9	4.4	3.9	79	93
Atrazine	0.6	0.5	2.3	2.5	82	96
Ametryn	5.1	2.2	5.9	3.1	86	93
Propazine	5.6	2.1	9.0	2.6	84	94
Terbuthylazine	4.1	1.9	5.6	2.4	86	95
Prometryn	3.1	1.6	16	2.6	84	94
Terbutryn	3.8	2.6	8.0	2.7	89	91

VALIDATION OF A MATRIX SOLID PHASE DISPERSION METHODOLOGY FOR THE DETERMINATION OF TRIAZINES HERBICIDES IN FISH

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ABSTRACT

A method using dual process columns of Matrix Solid Phase Dispersion (MSPD) and Solid Phase Extraction (SPE) has been validated for extracting and cleaning-up of nine triazine herbicides (ametryn, atrazine, cyanazine, prometryn, propazine, simazine, simetryn, terbuthylazine and terbutryn) in trout samples. For this purpose, freeze dried samples (0.2 g) were blended with 2 g of octadecylsilyl-derivatized silica (C_{18}) and transferred into a SPE cartridge containing ENVI^{TM}-Carb II/SAX/PSA (0.5/0.5/0.5 g) as clean up co-sorbent. Then the dispersed sample was washed with 10 mL of *n*-hexane and triazines were eluted with 20 mL ethyl acetate and 5 mL acetonitrile. Finally the extract was concentrated to dryness, re-constituted with 1 mL methanol and injected into the HPLC-DAD system. Recoveries varied between 88 and 105%, and repeteability and reproducibility were lower than 13%, meeting the requeriments stipulated by European Union legislation. The main advantages of this methodology when compared with conventional methods of sample preparation to screen herbicides in fish matrices are easy of work-up, fast, cheap, avoidance of clean-up procedure, as well as the reduction of organic solvents and energy requeriments in agreement with the principles of Green Chemistry.

1. Introduction

Fish can be harvested from waters that are contaminated by environmental chemicals, which may accumulate in fish at levels that can cause human health problems. Concern for these contaminants primarily focuses on fish harvested from aquaculture ponds, freshwater bodies, estuaries, and near-shore coastal waters (areas subject to shoreside contaminant discharges), rather than from the open ocean [1]. Environmental contaminants, such as pesticides, may also accumulate in aquacultured fish through contaminated feed ingredients [2]; furthermore, certain pesticides are applied directly to the water in aquaculture ponds to control weeds and algae and to eliminate invertebrates.

Nowadays there is a wide public interest in aquaculture. In fact, world aquaculture production is increasing much more rapidly than animal husbandry and capture fisheries, the other two sources of animal protein for the world population. There is widespread recognition that seafood production from capture fisheries is at or near its peak, and that aquaculture will become increasingly important as a source of seafood production [3]. Therefore the control of chemical groups which can originate important problems when reaching fish in farms is of a great interest.

Triazines are a group of herbicides that are present in the ten most-used herbicide formulations in Europe. Because of their relatively low soil adsorption and high solubility in water, these compounds migrate from soil to water [4] and due to the water cycle, fluxes of them reach seawater and affect the marine biota, having the coastal systems little capacity to degrade these compounds [5]. Furthermore, aquatic organisms are able to accumulate pesticide residues in much higher concentration than the surrounding water [6].

Therefore, triazines are considered as an important class of chemical pollutants and the Environmental Protection Agency (EPA) has proposed simazine and atrazine in the list of 67 pesticide for screening in the Endocrine Disruption Screening Program [7]. European Union has also included simazine and atrazine in the list of 33 priority substances in the European Union Water Framework Directive (2000/60/EC) [8], which committees the member states to achieve good qualitative and quantitative status of all water bodies, by way of Decision 2455/2001/EC [9]. Moreover, the Directive 2008/105/EC sets the Environmental Quality Standards (EQS) for these compounds in water and also shows the need to set EQS for these compounds in sediments and/or biota in order to protect the aquatic environment [10]. Since article 16 of the Water Framework Directive (2000/60/EC), demands a regular review of the priority substances, in January 2012, fifteen additional substances were proposed by the Commission (Proposal COM (2011)876) to be added to the list of priority substances [11]. Among these, terbutryn was proposed. Limit values have not established yet for triazines in fish and fishery products; however the U.S. Food and Drug Administration (FDA) has set a tolerance level of 12 mg kg^{-1} ww (wet weight) for simazine in the edible portion of finfish [1].

The sample preparation procedure is one of the most critical steps in analytical methods. In recent years, many innovations have been developed in the analytical processes applied to prepare food samples for the extraction and determination of pesticide residues. These methodologies include matrix solid phase-dispersion (MSPD), which combines aspects of several analytical techniques allowing sample homogenization, disruption, extraction, fractionation and clean-up within a single process [12]. In MSDP

the solid sample is blended in a mortar with an appropriate sorbent to obtain complete disruption and dispersion of the sample on the solid support. The blend is packed into a column from which the analytes are eluted with a relatively low solvent volume. Often, a co-sorbent material is placed at the bottom of the column to be filled with the blended sample to assist the extract clean up [13]. The key factors for the success of MSPD are its feasibility, flexibility, versatility, high throughput, low cost and rapidity. MSPD methods have been developed for the extraction of triazine residues from different plants and plant materials [14,15]; however references for the determination of triazines in animal tissues by MSPD are scarce and to the best of our knowledge, no studies using MSPD have been done to extract these chemicals residues from finfish.

Therefore, the aim of this work was the validation of an effective, simple and fast method for the simultaneous determination of nine triazine herbicides in fish based on Matrix Solid Phase Dispersion (MSPD) and Solid Phase Extraction (SPE) clean-up followed by High Performance Liquid Chromatography (HPLC) coupled to Diode array Detection (DAD). In this study, rainbow trout (*Oncorhynchus mykiss*) from aquaculture was selected because there has been a large increase in salmonid production, and unlike many of the major products from aquaculture, there is substantially more aquaculture production of salmonids in developed countries than in developing countries [16]. On the other hand, trout is one of the four typical representative commodities included in the category of fish to carry out procedures for pesticide residues analysis in food and feed by SANCO guidelines [17]; furthermore, FDA considers the aquaculture trout as a potential fish-related hazard by environmental chemicals [1].

2. Material and methods

2.1. Samples

1 kg of rainbow trout (*Oncorhynchus mykiss*) from aquaculture were purchased from a local market in A Coruña city (Northwest of Spain). Skin, bones and inner organs were discarded and muscle tissues were chopped, homogenized and freeze-dried. Then samples were homogenized again grounding it to a fine powder by an electrical mill and finally they were stored in glass bottles out of light exposure until analysis. The water content was determined gravimetrically by weighing before and after lyophilisation.

2.2. Chemicals

(a) *Herbicide standards*- Herbicides (ametryn, atrazine, cyanazine, prometryn, propazine, simazine, simetryn, terbuthylazine and terbutryn) analytical standards were supplied by Sigma-Aldrich (Inc. St. Louis, MO, USA). The individual stock standard solutions of 1000 mg L^{-1} were prepared in methanol by exact weighing of high-purity substances and stored at -18º C in the dark. Then a mixture of all the compounds was prepared in methanol containing 10 mg L^{-1} each individual triazine and stored at -18º C.

All working solutions were daily prepared by appropriate dilution of the 10 mg L^{-1} standard solutions with methanol.

(b) *Solvents* – *n*-hexane 95 % and methanol were superpurity Solvents from Romil (Cambridge, UK). Acetonitrile (ACN) (HPLC grade) and ethyl acetate (PAR, solvents for analysis of pesticide residues by GC) for instrumental analysis were from Panreac (Barcelona, Spain). Milli-Q water was obtained from a purification system from Millipore (Billerica, MA).

(c) *Sorbents* – *Bulk packing:* Supelclean™ Envi™-18 and *SPE tubes*: Supelclean™ Envi-Carb™ II/SAX/PSA (500 mg/500 mg/500 mg) were from Sigma-Aldrich (Inc. St Louis, MO, USA).

(d) *Filters* - Polytetrafluoroethylene (PTFE) filters of 0.45 µm were from Teknocroma (Barcelona, Spain).

2.3. Materials and apparatus

A Visiprep® vacuum distribution manifold from Supelco (Bellefonte, PA, USA) was employed in the purification step. A Büchi R-3000 rotary evaporator (Büchi Labortechnic AG, Flawil, Switzerland) was used in the evaporation step.

Chromatographic analyses were carried out in a high performance liquid chromatography-diode array detector (HPLC-DAD). The system consisted of a 2695 pump with a 996 Diode Array Detector from Waters (Milford, MA, USA). The column was a stainless steel column (150 mm x 4.6 mm ID, particle size 5 µm) packed with Hypersil GOLD C_{18} chemical bonded phase from Thermo Scientific (Austin, TX, USA).

2.4. Extraction procedure

Samples were extracted by following a procedure previously developed in our laboratory for determination of the target compounds in mussel samples [18], with a modification regarding the sample amount. Under final conditions, 0.2000 g of freeze-dried trout sample was homogenised with 2.00 g of Envi™-18 in a glass mortar with a pestle for 5 min. The final mixture was transferred into a 20-mL SPE cartridge containing a triple sorbent layer of 1.5g Supelclean™ Envi-Carb™-II/SAX/PSA (500/500/500 mg). Once packed, MSPD/SPE columns were connected to a Visiprep® vacuum distribution manifold and washed with 10 mL of hexane. Elution was performed with 20 mL of ethyl acetate and 5 mL of ACN and the obtained eluate was evaporated to a drop in rotary-evaporator and got to dryness by a gentle nitrogen stream. The residue was reconstituted in 1 mL methanol and the solution was filtered through of a 0.45 µm syringe filter of PTFE. As an illustration, figure 1 shows the scheme of the MSPD-SPE procedure.

2.5. HPLC-DAD conditions

The chromatographic analysis was carried out using the following ACN:H_2O gradient elution: ACN initial percentage of 30 % (8 min), increased linearly to 40 % in 5 min; increased to 50 % in 5 min, after which the percentage was returned to the initial conditions in 9 min. A constant mobile phase flow rate of 1 mL min^{-1} and 20 µL of sample volume were used.

The absorbance was measured continuously in the 200-400 nm range and peaks areas quantification were carried out at 222.7 nm in order to achieve maximum sensitivity. All triazine herbicides were identified initially by retention time and then by spectral identification contrasting the spectrum with a standard library created in the same wavelength interval.

3. Results and discussion

3.1 MSPD application

Sorbent selection is of utmost importance since it is one of the variables controlling the selectivity of MSPD processes [19,20]. In this work, we applied a method which employs C18 as dispersant, Envi-Carb II/SAX/PSA as clean-up co-sorbent and a mixture of ethyl acetate and acetonitrile as elution solvent. C18 is by far the most popular sorbent, especially for analyte extraction from animal. Regarding the tri-layer SPE cartridge, ENVI-Carb II is a graphitized non porous carbon that has a strong affinity towards planar molecules and can remove pigments and sterols, PSA is a polymerically bonded ethylenediamine-N-propyl phase that contains both primary and secondary amines, that retains fatty acids, organic acids, sugars and some polar pigments, and SAX is a quaternary amine that offers additional ion exchange capacity for removing matrix components.

During extraction of complex matrices, multiple unwanted compounds from the matrix are extracted along with the compounds of interest. A major issue when dealing with the chromatographic determination of organic compounds in fish samples is to minimize the lipid content of the extracts [21,22]. If lipids are not removed in the sample preparation process, they may cause retention time shifts, analyte peak distortion, decrease of sensitivity and reduction of column life, thus compromising quality of analysis. Therefore the determination the pesticides in food is often complicated by the presence of different fat content [23].

First assays were carried out employing 0.5000 g of trout sample following the procedure previously described for determination of triazines in mussel samples [18]. After evaporation of the eluates, it could be seen that the extracts have an appreciable amount of lipid material, and were not analysed further. This can be explained because

of the higher lipid content in trout (4.8%) than in mussel (2.2%) [24]. Therefore, the following experiments were carried out using 0.2500 g of trout sample. In this case, after evaporation of eluates, extracts were clean enough to be processed; however after redissolution on methanol and filtration through PTFE filters, it could be observed some small particles of fat, thus the extracts were not analysed. Finally, considering 0.2000 g of trout sample, the extracts obtained were clean enough to be injected into the HPLC system. As an example, figure 2 shows the chromatogram corresponding to a trout sample extracted and purified under the considered procedure; as it can be seen, none of the triazines under study were detected.

3.2 Method validation

The method was validated in terms of accuracy, precision and limits of quantification according to "validation parameters and criteria" from SANCO Guidelines for method validation and quality control procedures for pesticide residue analysis in food and feed [17]. Quantitative results were calculated using matrix matched standards prepared by spiking the final extracts from blank samples of trout samples with different levels of triazines. The linearity of the calibration curves was calculated at a concentration range between 0.5-5 mg kg^{-1} dried sample by duplicate analysis at five different concentration levels. Excellent linearities were obtained with coefficients of determination (R^2) higher than 0.998 for all triazines.

The accuracy and precision of the proposed method were determined by analysis of five replicates of uncontaminated trout sample spiked at two concentration levels (0.500 and 5.00 mg kg^{-1} dried sample, equivalent to 0.125 and 1.25 mg kg^{-1} fresh sample, respectively). The obtained results demonstrated that the method achieved satisfactory recoveries for all compounds in the range of 88-105 % for the low level and of 96-100 % for the high level with associate standard deviations below 8.6% and 2.5 % respectively (Table 1). These values values are in the acceptance range of the DG SANCO/12571/2013 of the European Quality Control Guidelines (mean recoveries from 70 to 120% and RSD $\leq$20%), which indicate that the method meets the requirements stipulated. As an example, figure 3 shows the chromatograms corresponding to spiked trout sample, fortified at both levels.

The limit of quantification (LOQ) is defined as the lowest concentration of the analyte which has been validated meeting the method performance acceptability criteria (i.e. the average recovery was in the range 70-120% with RSD $\leq$20%) and was determined based on the accuracy and precision data obtained through the recovery studies. As it can be seen on Table 1, 0.5 mg kg^{-1} meets the method performance acceptability criteria for trueness and precision by applying the complete analytical method (DG SANCO/12571/2013). On the other hand, the reduction in the weight of trout samples

during freeze-drying accounted for ca. 75% (0.2 g of freeze-dried sample equivalent to ca.0.8 g of fresh tissue); therefore, approximately 4-fold lower LOQ values are achieved when referred to fresh sample.

4. Conclusions

The suitability of a procedure based on MSPD and SPE for the extraction of nine triazines from finfish samples has been demonstrated for the first time. The method uses Envi^{TM}-18 as dispersant with Envi-Carb^{TM}II/SAX/PSA co-column followed by a combination of 20 mL ethyl acetate and 5 mL acetonitrile as elution solvent. The developed method provides satisfactory accuracy and precision for the determination of triazines in aquaculture trout samples. The main advantages of this methodology when compared with classical methods of sample preparation to determine triazines in animal tissues are easy of work-up, fast, low cost, avoidance of clean-up procedure, as well as the significant reduction of organic solvents and energy requirements in agreement with the principles of the Green Chemistry.

Acknowledgements

This work has been supported by Ministerio de Economía y Competitividad (CTM2013-48194-C3-2-R) and Program of Consolidation and Structuring of Units of Competitive Investigation of the University System of Galicia (Xunta de Galicia) potentially cofounded by European Regional Development Fund (ERDF) in the frame of the operative Program of Galicia 2007-2013 (reference: GRC2013-047). N. Rodríguez-González also thanks the "Xunta de Galicia" and Campus do Mar International Campus of Excellence for the concession of her PhD. grant.

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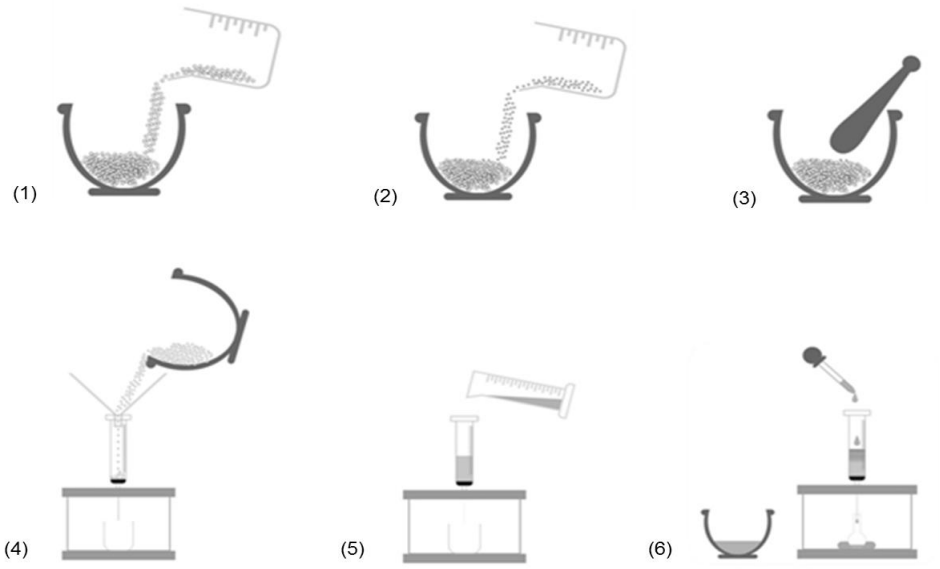


Figure 1. Scheme of the conditions of MSPD-SPE procedure. (1) 2.00 g C18; (2) 0.2000 g freeze-dried trout; (3) Homogenization; (4) Transfer to a clean-up co column with EnviCarb/SAX/PSA (500/500/500mg); (5) Rinsing with 10 mL hexane; (6) Elution with 20 mL ethyl acetate and 5 mL acetonitrile

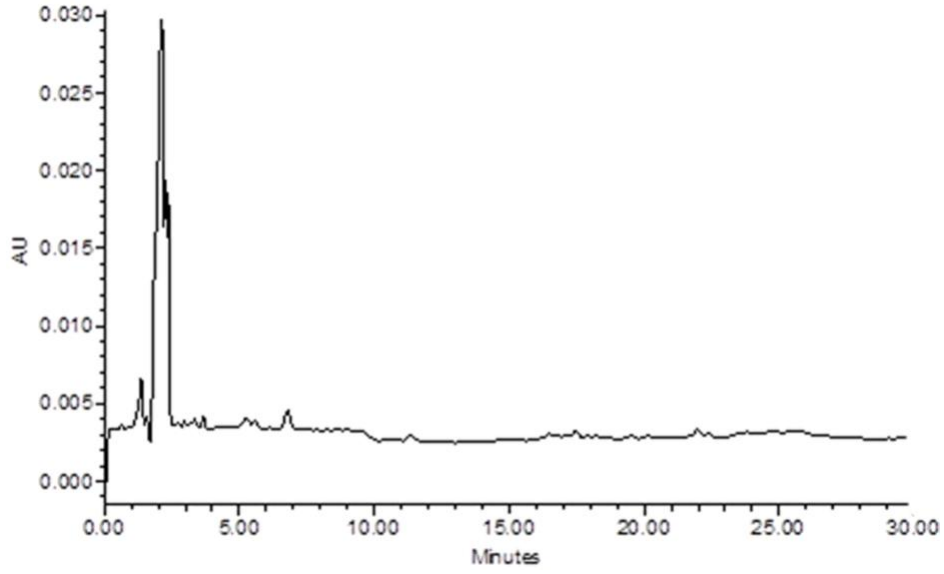
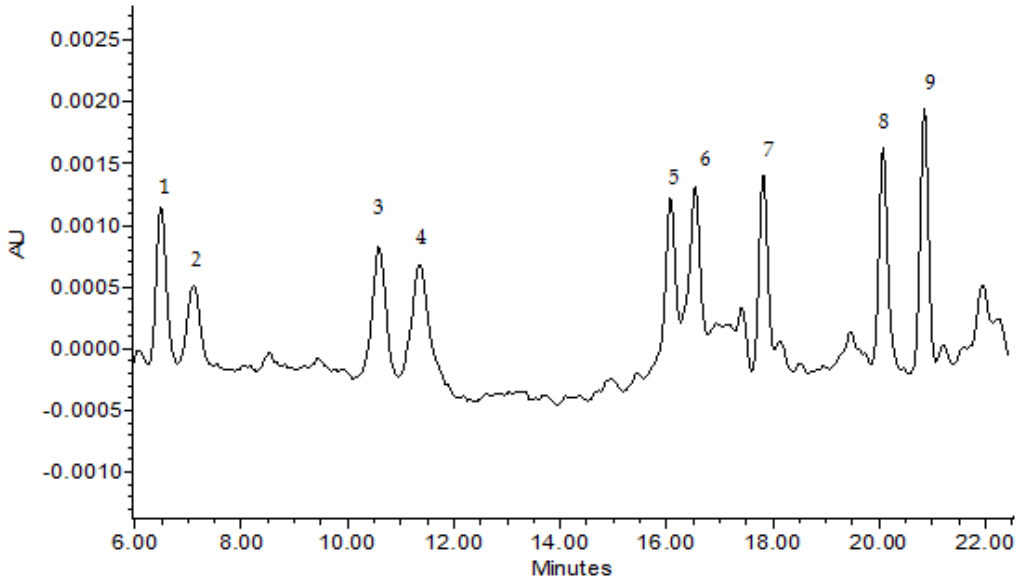


Figure 2. HPLC-UV chromatogram of a trout extract obtained by MSPD

(a)



(b)

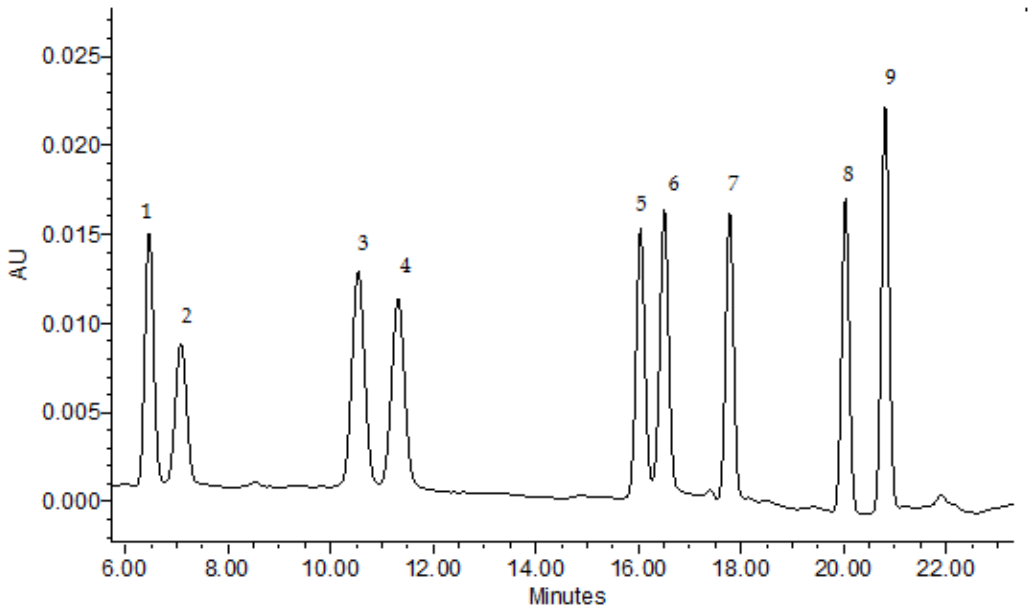


Figure 3. HPLC-UV chromatograms of a spiked trout sample obtained by MSPD. (a) Trout sample spiked at 0.5 mg kg^{-1}; (b) trout sample spiked at 5 mg kg^{-1}. Target compounds are numbered as follows: (1) Simazine, (2) Cyanazine, (3) Simetryn, (4) Atrazine, (5) Ametryn, (6) Propazine, (7) Terbuthylazine, (8) Prometryn, (9) Terbutryn

Table 1. Precision and accuracy of target compounds in trout samples with MSDP-HPLC-DAD (n=5)

	Analytical recovery (%)	± RSD (%)
Compound	0.5 mg kg^{-1}	5 mg kg^{-1}
Simazine	20 ± 3.8	97 ± 1.4
Cyanazine	105 ± 5.9	98 ± 1.5
Simetryn	90 ± 6.7	97 ± 2.0
Atrazine	101 ± 4.9	99 ± 2.5
Ametryn	96 ± 4.5	96 ± 2.3
Propazine	101 ± 8.6	98 ± 1.6
Terbuthylazine	88 ± 6.7	100 ± 1.4
Prometryn	95 ± 3.8	100 ± 1.1
Terbutryn	100 ± 4.7	100 ± 1.6

CAPÍTULO V

DISEÑO DE UNA ACTIVIDAD DOCENTE

INTRODUCCIÓN

En el capítulo V se diseña una actividad docente, en la que se aplica uno de los métodos desarrollados en la Tesis Doctoral a una práctica de laboratorio tanto para alumnos del Grado en Química como para estudiantes de Ciencias Ambientales. La actividad consiste en la determinación de cuatro triazinas (Atrazina, Propazina, Simazina y Terbutilazina) en agua de mar, utilizando para su determinación cromatografía de líquidos de alta resolución con detector ultravioleta (HPLC-UV).

El principal objetivo pedagógico de las sesiones de laboratorio consiste en el aprendizaje del uso del instrumento HPLC-UV así como de los principios de las medidas cromatográficas.

Además este proyecto permite a los alumnos practicar y desarrollar un elevado número de destrezas incluyendo la preparación de disoluciones, extracción en fase sólida y el empleo de distintos métodos de concentración de extractos de muestras. Adicionalmente, los alumnos adquieren experiencia en procedimientos y seguridad en el laboratorio y en la eliminación de residuos, familiarizándose al mismo tiempo con algunos de los principios de la Química Verde.

Publicaciones:

Optimization of a HPLC-UV method for the analysis of Chloro-s-triazines in seawater samples. González-Castro, M.J., Rodríguez-González, N., Beceiro-González, E. (2016). *Current Topics in Analytical Chemistry*. 10, 23-28. **Anexo I-VII, página 341.**

Congresos:

Determination of triazinic herbicides in seawater samples by high performance liquid chromatography. (2015). Rodríguez-González, N., Beceiro-González, E., González Castro, M.J. *XXXV Bienal RSEQ*. (ISSN: 978-84-606-9786-2). Página 260. A Coruña. **Anexo II-IV, página 371.**

OPTIMIZATION OF A HPLC-UV METHOD FOR THE ANALYSIS OF CHLORO-S-TRIAZINES IN SEAWATER SAMPLES

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ABSTRACT

A student activity that focuses on the determination of four triazines (atrazine, propazine, simazine and terbuthylazine) in sea water samples by using High Performance Liquid Chromatography with Ultraviolet Detection (HPLC-UV) is presented. During the laboratory sessions students learn both the use of the instrument and the principles of chromatographic measurements by testing several mobile phases and applying the optimized method to the analysis of surface waters. Additionally, this project allows students to practice and develop a number of skills including solution preparation; solid phase extraction and sample concentration procedures. This laboratory experiment is suitable for both analytical chemistry and environmental sciences and the use of this method as undergraduate experiment permits to introduce concepts of green chemistry.

Keywords: *Undergraduate Laboratory Instruction, Analytical Chemistry, HPLC, Hands-On Learning/Manipulatives, Environmental Science, Triazines.*

1. Introduction

Triazines are well known herbicides that are applied worldwide to soil for the control of weeds in many agricultural fields, as well as, railway lines, roadsides and golf courses. Because of their relatively low soil adsorption and high solubility in water, these compounds migrate from soil to water [1] and as a result of the water cycle, fluxes of these compounds reach seawater. As the coastal systems have little capacity to degrade these compounds [2], biota from marine environment is affected [3, 4].

The mechanism of action of triazines is *via* photosynthetic inhibition, and for this reason, they are only toxic for plants; however these compounds can affect human health through the dietary intake. These compounds are highly persistent and can survive many years in soil, water and organisms. Therefore, they are considered as an important class of chemical pollutants, and the U.S. Environmental Protection Agency (EPA) has included simazine and atrazine in the list of 67 pesticides identified for the

Endocrine Disruption Screening Program [5]. European Union has also included simazine and atrazine in the list of 33 priority substances covered under the EU Water Framework Directive [6], which requires the member states to achieve good qualitative and quantitative status for all water bodies, by way of Decision 2455/2001/EC [7]. Furthermore, the presence of pesticides in surface waters is regulated by the European Directive 2008/105/EC [8] that establishes a maximum permitted concentration of 2 and 4 $\mu g\ L^{-1}$ for atrazine and simazine, respectively.

The determination of triazines is currently carried out by chromatographic techniques, mainly by Reversed Phase High Performance Liquid Chromatography (RP-HPLC), generally employing the mixture acetonitrile-water as mobile phase. Regarding the detection system, the detectors used are ultraviolet [9, 10], diode array [11, 12] or mass spectrometry [13]. For this project we have chosen UV detector because it is relatively inexpensive and also commonly found in teaching labs.

High performance liquid chromatography is an integral part of any analytical chemistry unit; in our laboratory there is a strong focus on HPLC as it is a technique commonly valued and looked for in our graduates when they pursue for academic and/or industry positions. Therefore, the present experiment has been designed to show the applications of reversed-phase chromatography and to train students in the use of high performance liquid chromatography by optimizing HPLC-RP methods and analyzing the presence of triazines in sea water samples. Chromatographic techniques are very important in analytical chemistry and environmental sciences; thus the project described is meant to be used as a demonstration whereby the students can understand the overlap among different fields of chemistry.

On the other hand, an extraction procedure to preconcentrate the analytes and remove possible interferences is mandatory to achieve the maximum permitted levels established for these pollutants in seawater samples by the European Directive. For this purpose, we have chosen solid phase extraction (SPE) because it is the preconcentration technique most commonly used for the determination of triazines in water samples [13, 14, 15].

Therefore the main aim of this work is the employment of a simple, effective and low-cost method for the determination of four triazine herbicides in seawater samples based on SPE followed by HPLC-UV as a laboratory experiment for undergraduate students. Thus this experiment acquaints students on a number of essential techniques in the analytical chemistry laboratory. Furthermore, the students would get hands-on experience on laboratory safety procedures and on waste disposal.

2. Materials and methods

2.1. Activitiy schedule

Before performing the laboratory sessions, students are required to complete a prelab exercise that assesses their preparedness to perform the experiment. They should to be able to describe the configuration of a HPLC system and what experimental parameters are expected to be important [16, 17]. The prelab exercise must be shown to the instructor at the beginning of the laboratory sessions.

The experiment is divided into two 3-h lab periods and one 2-h classroom period. In the laboratory sessions the students work as a group of six which allows to generate stimulating discussions between the members of the group. In the first lab period, students get acquainted with the HPLC technique (components of HPLC system and developing of HPLC-UV methods). In the second lab period they prepare the seawater samples by SPE and the standard solutions for calibrations, and then run the equipment to measure the concentration of triazines.

Once completing the laboratory periods the students are required to make a report on the obtained results. Finally, a classroom session with all groups of students is carried out for a postlab discussion about the whole procedure.

2.2. Chemicals and materials

Herbicide analytical standards (atrazine, propazine, simazine and terbuthylazine) were supplied by Sigma-Aldrich. A solution containing all the studied triazines at a concentration of 10 µg mL^{-1} in methanol was prepared previously by the instructor. For quantitation of the target analytes linear calibration curves over 5 concentration levels (0.4, 0.6, 0.8, 1 and 2 µg mL^{-1}) were prepared. Acetonitrile (ACN) was purchased from Panreac and methanol and acetone from Romil. Ultra-pure Milli-Q water was obtained *via* a Millipore Milli-Q system (Millipore). Reverse phase polymeric cartridges Oasis HLB (6 mL, 200 mg) were supplied by Waters.

2.3. Hazards

Triazines (atrazine, simazine, propazine and terbuthylazine) are irritants to skin, eye and respiratory tract. Therefore, when handling them dispersion of dust should be avoided. Acetonitrile and methanol are highly flammable and can cause skin and eye irritation. They must be kept away from sources of ignition, heat surfaces and strong oxidizers. While using all these substances personal protective equipments, such as gloves and goggles, must be worn at all times.

2.4. Chromatographic procedure

The HPLC equipment consisted of 1100 Agilent chromatograph equipped with a pump, an ultraviolet detector, a 20 µL Rheodyne injection loop and a computer that runs on

ChemStation data processor. For separation an Envirosep PP column (125mm x 4.6 mm ID, particle size 5 µm) from Phenomenex was employed.

Isocratic elution was performed with acetonitrile:water at several volume ratios (70:30, 55:45 and 45:55). The mobile phase flow rate was 0.8 mL/min in the three eluting regimes. Solvents were desgassed by a low flow of helium. The absorbance was measured at 223 nm in order to achieve maximum sensitivity.

2.5. Solid Phase Extraction procedure

Samples were extracted by following a procedure previously developed in our laboratory for the determination of the target compounds in seawater samples [15]. In summary it consists of the following steps: Oasis HLB cartridges were connected to a Visiprep® vacuum distribution manifold (Supelco). Prior to their use cartridges were conditioned by washing with 10 mL methanol and 10 mL Milli-Q water. Seawater sample (50 mL) was pumped through the cartridge at a flow rate of 10 mL min^{-1} and then the cartridges were washed with 20 mL Milli-Q water. Once the retention step had been completed, the cartridge was partially dried under a vacuum system for 5 min and totally dried using a nitrogen stream for 30 min. The elution of retained compounds was done with 3 mL of acetone and the organic extract was brought to complete dryness through a combination of rotary evaporator (Büchi, Labortechnic AG) at 40 ºC and a gentle nitrogen stream. Finally the sample was reconstituted in methanol/water (1:1, v/v) to a final volume of 1 mL and injected into the HPLC.

2.6. Treatment of residues

All solutions and HPLC eluent were discarded in an organic waste container. Gloves and tips from micropipettes were collected in a plastic residue container. Pasteur pipettes were disposed in a glass residue container.

3. Results and discussion

3.1. Optimization of the HPLC method

The triazine structure consists of a six-membered aromatic heterocyclic containing three nitrogens in the ring. The triazine herbicides currently used are mostly *1,3,5*-triazines (symmetrical triazines) which have a chlorine, thiomethyl or methoxy group at the 2-position of the ring and are usually substituted in the 4 and 6 positions with alkylamino groups [18].

For reversed phase partition mode the separation depends on the average hydrophilic or hydrophobic character of a compound. The components more soluble on the eluent are eluted earlier from the column. Triazines have both hydrophilic and hydrophobic functionalities spatially separated. The electron pairs on the N-atom rings form hydrogen bonds with water molecules and thus triazine rings are hydrophilic. However,

the alkylamino chains in the 4 and 6 positions are hydrophobic [19]. The lipophilic/hydrophilic character of a substance is represented by octanol/water partition coefficient (Kow) which is an important physicochemical descriptor for assessing transport pathways and environmental partitioning of herbicides [20]. In this work we determined four chloro-s-triazines whose structures and recommended log Kow [21, 22] are shown in figure 1. As it can be seen, the hydrophobicity increases with increasing length of the alkyl chains; therefore the elution order is simazine, atrazine, propazine and terbuthylazine.

The most widely used strategy for the development of a RP-HPLC method consists in starting at a high percentage of the organic component in the mobile phase and making stepwise changes in the solvent strength until the separation looks good. Then the percentage of each component in the mobile phase would be fine-tuned in smaller steps [23]. Therefore initially isocratic elution with a ratio 70% ACN:30% H_2O was carried out; at this ratio the peaks are distinguished but are not fully separated. At 55% ACN:45% H_2O the peaks of simazine and atrazine are adequately resolved but propazine and terbuthylazine remain partially overlapped. At 45% ACN:55% H_2O good resolution of the four triazines is achieved. Thus this elution ratio was chosen for the qualitative and quantitative analysis of a surface water sample. Figure 2 shows the chromatograms obtained at the three elutions regimes. As it can be seen, three changes can be observed as a result of the decrease in solvent strength: longer retention times, the overall separation tends to improve and peaks become broader and shorter.

Once the optimal mobile phase was selected, the five standard solutions were injected. Calibration curves were constructed using least-squares regression of concentration versus the peak area of the calibrations standards. Then a seawater sample was analysed.

3.2 Laboratory experience for undergraduate students

Our main aim was to design a laboratory experiment that allows students to participate actively in the learning of concepts through practical experience. It increases the student motivation and makes teaching the principles involved in chromatographic techniques easier. On the other hand, future chemists must know the necessary tools to support and promote global sustainability [24, 25], which lead to safer and more efficient work environments on the laboratory. Therefore green chemistry principles have been incorporated at the laboratory classes. Furthermore, the procedure described herein is meant to be used as a demonstration whereby the students can understand the overlap among different fields of chemistry.

The four pedagogical goals of this experiment were: 1) Introduction to the HPLC-UV instrumentation (components of HPLC system and optimization of HPLC-RP methods);

2) Learning of SPE procedure as a sample pretreatment technique as well as the concentration step; 3) Qualitative and quantitative analysis; and 4) Evaluation of the process taking into account the principles of green chemistry (waste generation, safety of processing steps, health and environmental impact of the reagents, etc.). The experiment also opens the discussion of other concepts such as absorbance, chemical functionality (hydrophobibicity/philicity, toxicity, etc.), and state-of-art about legislation of triazines in sea water.

After finishing the laboratory sessions the students are required to make a report on the obtained results, which will help the students to develop the scientific writing skills they would need later in their careers. This report requires students to interpret and analyse their data and also to answer a list of questions on the RP-HPLC-UV technique.

Finally, after all students in the course have completed the laboratory periods, they are required to present their results in a classroom session. This will instigate interesting discussions because the different groups of students have to compare the results obtained, to formulate explanations about the experimental procedure carried out, and also propose solutions to obstacles or undesirable outcomes. They also evaluate the whole procedure taking into account the principles of green chemistry.

4. Conclusions

This project is suitable for a typical undergraduate analytical laboratory course and provides students a good opportunity to understand the connection between analytical chemistry and environmental sciences. The experiment is mainly focused to train students in the use of high performance liquid chromatography by optimizing HPLC-RP methods. Additionally this activity acquaints students on preparation of solutions, solid phase-extraction of herbicide residues from liquid samples and employment of concentration steps. Therefore it permits the students to gain experience on a number of essential techniques in the analytical chemistry laboratory. Furthermore they also get hands-on experience on laboratory safety procedures and familiarity with some of the principles of green chemistry. This activity is simple, cost-effective, and only requires readily available apparatus.

Acknowledgments

This work was financially supported by Xunta de Galicia potentially co-funded by European Regional Development Fund (EDRF) and by the Spanish Ministry of Economy, and Competitiveness co-funded by ERDF (CTM2013-48194-C3-2-R). N. Rodríguez-González also thanks the “Xunta de Galicia” for the concession of her Ph.D grant under the program of the International Campus of Excellence “Campus del Mar”.

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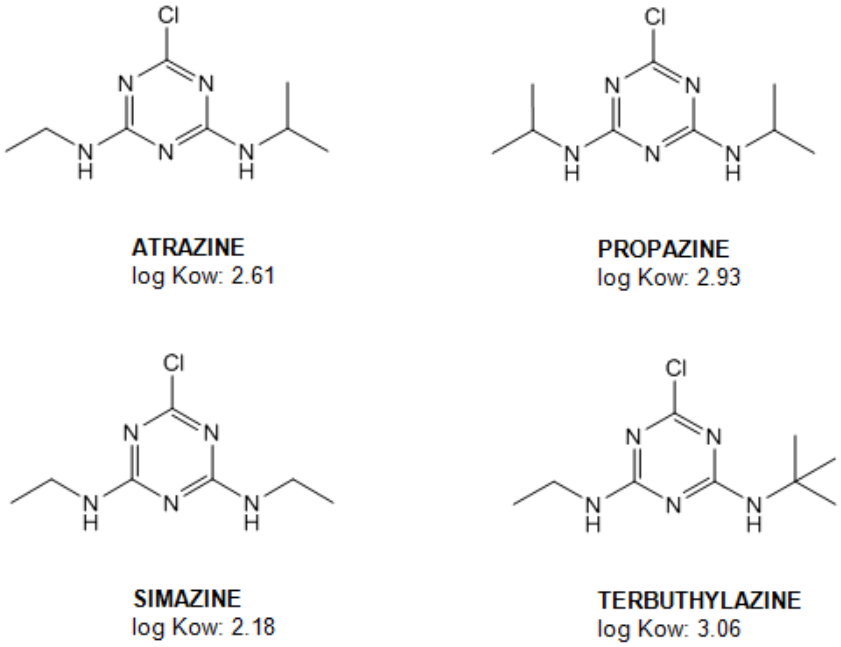

Figure 1. Structures and recommended log Kow of chloro-*s*-triazines.

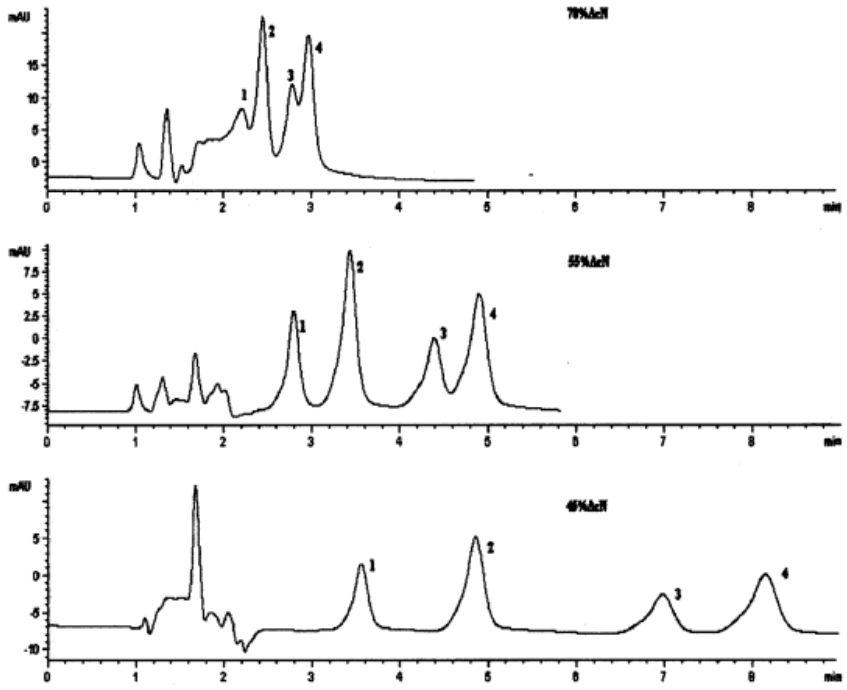

Figure 2. Chromatogram showing separation of triazines at three elution regimes. Compounds are numbered as follows: (1) Simazine, (2) Atrazine, (3) Propazine, and (4) Terbuthylazine.

DETERMINATION OF TRIAZINIC HERBICIDES IN SEAWATER SAMPLES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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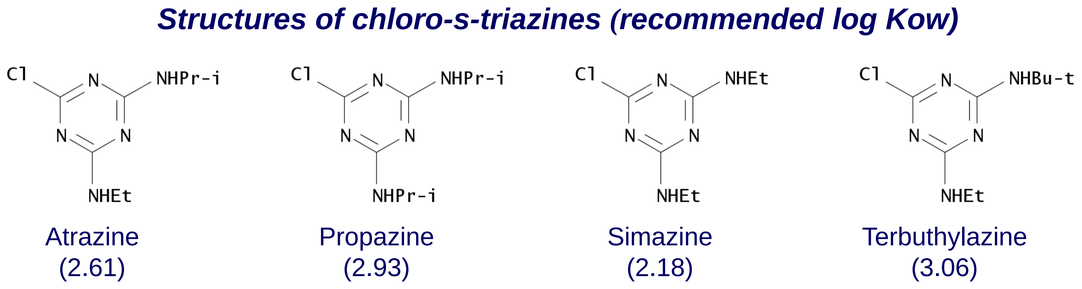
INTRODUCTION

A student activity focused on the determination of four chloro-s-triazines (atrazine, propazine, simazine and terbuthylazine) in sea water samples by using High Performance Liquid Chromatography with Ultraviolet Detection (HPLC-UV) is presented. This project allows students learn both the use of the instrument and the principles of chromatographic measurements by testing several mobile phases and applying the optimized method to the analysis of surface waters. Additionally, it is useful for the practice and development of a range of skills including solution preparation, solid phase extraction (SPE) and sample concentration procedures.

This laboratory experiment is suitable for both analytical chemistry and environmental sciences and the use of this method as undergraduate experiment permits to introduce concepts of green chemistry.

EXPERIMENTAL METHOD

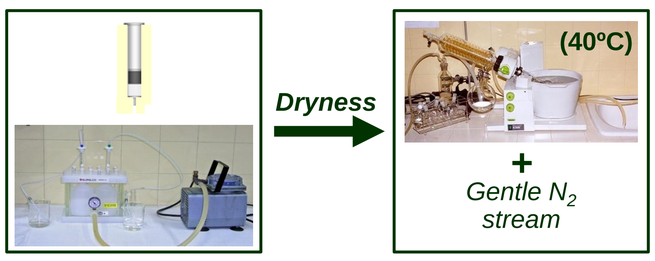
Structures of chloro-s-triazines (recommended log Kow)



Atrazine (2.61) | Propazine (2.93) | Simazine (2.18) | Terbuthylazine (3.06)

SPE Procedure

Cartridge Oasis HLB (200 mg) + 50mL water sample
3 ml acetone elution solvent



Dilution
Methanol : water (1:1)
1 mL

HPLC-UV Determination

HPLC-UV 1100 Agilent
Envirosep PP column
Absorbance: 223 nm

Isocratic elution ACN:H_2O
Volume ratios (70:30, 55:45 and 45:55)
Flow rate 0.8 mL/min

TEACHING METHODOLOGY

Activities Schedule

1. Prelab exercise

Assess preparedness

- Be able to describe the HPLC system
- Know experimental parameters [1, 2]

Show to the instructor before lab sessions

2. Lab sessions

Divided in two 3 h lab sessions

First lab session

Students acquaint with HPLC technique:
- Components of HPLC system
- HPLC-UV methods development

Second lab session

- Carry out SPE procedure
- Prepare standard solutions for calibrations
- Measure the concentration of triazines

Discussion between members of the group

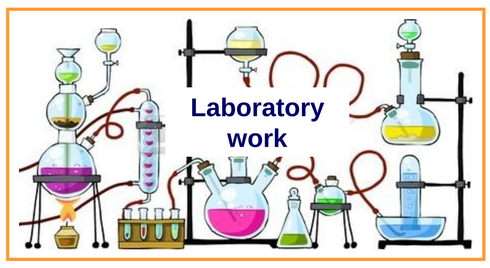
Make a report with obtained results and questions

3. Classroom session

Compare and explain the results of different groups

Discussion about :
- whole procedure
- green chemistry principles
- overlap among different chemistry fields
- concepts (absorbance, …)
- legislation

Laboratory work

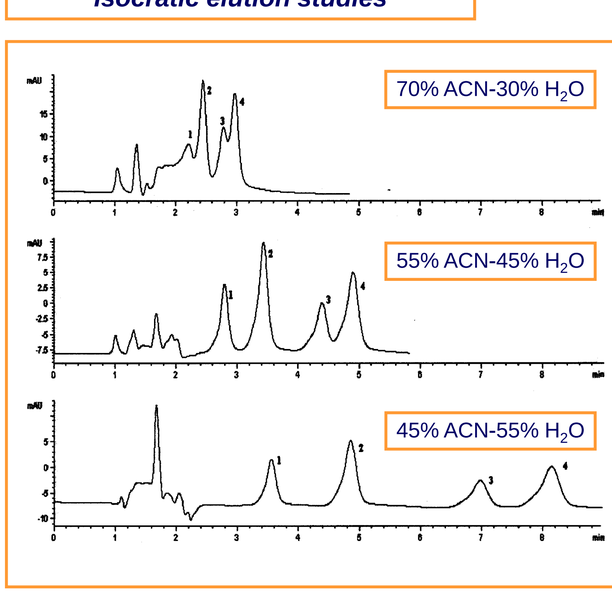


The experiment has being designed:
- to train students on the use of HPLC and SPE
- to show the applications of HPLC-RP to analyze triazines in sea water
- to treat the residues (solutions, gloves, tips from micropipettes and Pasteur pipettes)

Optimization of the HPLC method

Strategy
starting at high % of organic component in mobile phase and decrease the solvent strength until separation [3]

Isocratic elution studies



Peaks are distinguished but not fully separated

Propazine and terbuthylazine remain partially overlapped

Good resolution of the four triazines
Longer retention times
Peaks become broader and shorter

Elution ratio for water samples application

Chromatogram showing separation of triazines at three elution regimes
(1) simazine, (2) atrazine, (3) propazine, (4) terbuthylazine

Analysis of seawater sample

- Calibration curves were constructed
- Extraction procedure was carried out
- Seawater sample was analysed.

Laboratory experience for undergraduate education

The laboratory experiment designed allows students to participate actively in learning of concepts through practical experience

Pedagogic goals

1) Introduction to the HPLC-UV
2) Learning of SPE procedure
3) Qualitative and quantitative analysis
4) Waste generation
5) Safety of processing steps
6) Health and environmental impact of the reagents
7) Develop the scientific writing skills (report)
8) State of art about legislation of triazines in water

This experience increases student motivation and makes teaching HPLC easier

Future chemists must know the necessary tools to support and promote global sustainability which lead to safer and more efficient work environments on the laboratory

CONCLUSIONS

This project is suitable for a typical undergraduate analytical laboratory course and provides students a good opportunity to understand the connection between analytical chemistry and environmental sciences. The experiment is mainly focused to train students in the use of high performance liquid chromatography by optimizing HPLC-RP methods. Additionally this activity acquaints students on preparation of solutions, solid phase-extraction of herbicide residues from liquid samples and employment of concentration steps. Therefore it permits to the students gain experience on a number of essential techniques in the analytical chemistry laboratory. Furthermore they also get experience on laboratory safety procedures and familiarity with some of the principles of green chemistry. This activity is simple, low cost, and requires readily available apparatus.

Acknowledgments
The authors acknowledge the financial support of the “Xunta de Galicia” by Program of Consolidation and Structuring of Units of Competitive Investigation of the University System of Galicia potentially cofounded by ERDF in the frame of the operative Program of Galicia 2007-2013 (reference: GRC2013-047). N. Rodríguez-González also thanks to the “Xunta de Galicia” for the concession of her award under the program “Campus del mar”

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CONCLUSIONES/CONCLUSIÓNS/CONCLUSIONS

Los herbicidas triazínicos constituyen uno de los grupos de herbicidas más empleados a nivel mundial. Estos compuestos son tóxicos y resistentes pudiendo permanecer muchos años en el suelo, el agua y los organismos. A pesar de ser persistentes, pueden ser degradados dependiendo de las condiciones químicas y biológicas del medio en el que se encuentren, dando lugar a metabolitos que también pueden presentar efectos tóxicos. Por ello alguno de estos herbicidas han sido legislados, mientras que otros y los productos de degradación todavía no se encuentran regulados. Tras su aplicación, a través del ciclo hidrológico, estos compuestos llegan al medio marino, distribuyéndose en todos sus compartimentos (agua, sedimentos, flora y fauna) en función de las propiedades de éste y de la naturaleza de las fases adsorbentes.

El medio marino es un patrimonio muy valioso que tiene que ser protegido, conservado y rehabilitado para que los océanos y mares estén limpios, siendo de este modo sanos y productivos. Por esta razón se necesitan más estudios sobre el comportamiento, efectos y presencia de estos compuestos en este medio. Para ello, se requieren métodos analíticos que permitan su determinación a bajos niveles de concentración que sean eficaces, precisos, sencillos y rápidos. En esta Tesis se ha desarrollado metodología analítica para la determinación simultánea de nueve herbicidas triazínicos (Ametrina, Atrazina, Cianazina, Prometrina, Propazina, Simazina, Simetrina, Terbutilazina y Terbutrina) y ocho de sus principales productos de degradación (Desetil-Atrazina, Desetil-Desisopropil-Atrazina, Desetil-2-Hidroxi-Atrazina, Desetil-Terbutilazina, Desisopropil-Atrazina, Desisopropil-2-Hidroxi-Atrazina, 2-Hidroxi-Atrazina y 2-Hidroxi-Terbutilazina) en distintos compartimentos del ecosistema marino. Cabe destacar que en la determinación de estos compuestos se han empleado desde técnicas analíticas convencionales a técnicas más sofisticadas y novedosas, las cuales han sido empleadas por primera vez bien para estos compuestos o para las matrices estudiadas. Debido a las ventajas que presentan y a los resultados satisfactorios obtenidos, los métodos propuestos mejoran aquellos desarrollados previamente con este propósito.

En lo que respecta a las técnicas instrumentales de análisis, se han optimizado y validado tres métodos cromatográficos: cromatografía de líquidos de alta resolución con detección con red de diodos (HPLC-DAD), cromatografía de líquidos con detección por espectrometría de masas en tándem (LC-MS/MS) y cromatografía de líquidos de ultra resolución con detección por espectrometría de masas en tándem (UPLC-MS/MS).

En primer lugar se desarrolló un método de HPLC-DAD para la determinación de las nueve triazinas, empleando C_{18} como fase estacionaria y elución en gradiente con acetonitrilo-agua, lográndose una buena separación y resolución para las nueve triazinas estudiadas. La absorbancia se midió en el rango de 200-400 nm y la cuantificación se

realizó a 222,7 nm con el fin de conseguir la mayor sensibilidad. Es importante señalar que no existen trabajos en la literatura en los que se logre una adecuada resolución de las nueve triazinas empleando esta técnica; además, en la mayoría de los artículos encontrados, Cianazina y Simazina presentan un solapamiento total cuando se emplea acetonitrilo en la fase móvil.

A continuación se puso a punto un método LC-ESI-MS/MS para la determinación de las nueve triazinas y sus ocho principales productos de degradación, empleando como modo de adquisición MRM (multiple reaction monitoring), debido a su sensibilidad y selectividad, C_{18} como fase estacionaria y elución en gradiente con acetonitrilo-disolución acuosa al 0,3% de ácido acético. La optimización de las condiciones de MS/MS incluyó la optimización del voltaje de cono y la energía de colisión para cada pesticida, con el fin de obtener el ion precursor y los productos correspondientes. Es importante resaltar que no se han encontrado referencias en las que se realice la determinación simultánea de los diecisiete compuestos estudiados.

Finalmente, en cuanto a técnicas instrumentales se refiere, se ha optimizado un método de UPLC-MS/MS para la determinación de las nueve triazinas y sus ocho productos de degradación, empleando C_{18} enlazada a partículas híbridas con puentes de etileno (BEH) como fase estacionaria y elución en gradiente con metanol-agua, conteniendo cada una de las fases móviles acetato amónico 5 mM como modificador. El empleo de columnas cortas empaquetadas con pequeño tamaño de partícula presenta como ventajas, respecto a la cromatografía de líquidos convencional, un menor tiempo de análisis así como de consumo de disolvente, mayor eficiencia de los picos cromatográficos y mejoras en los límites de detección. Por otro lado, es la primera vez que se emplea esta técnica para la determinación simultánea de los diecisiete compuestos objeto de este estudio.

En relación a los métodos de extracción, se ha optimizado y validado diferente metodología analítica en agua de mar, sedimentos y biota (algas, mariscos y pescados). En el caso del agua de mar se han optimizado y validado tres procedimientos analíticos: extracción en fase sólida fuera de línea (SPE off-line), extracción en fase sólida en línea (SPE on-line) y microextracción líquido-líquido dispersiva (DLLME). Para todos ellos se han obtenidos los Estándares Medioambientales de Calidad (EQS) de la legislación vigente en el momento del estudio.

En primer lugar se desarrolló un método de SPE off-line para la determinación de las nueve triazinas empleando DAD como sistema de detección. El método consiste en la extracción y preconcentración de 50 mL de muestra de agua empleando cartuchos HLB, lavado de interferencias con 20 mL de agua Milli-Q y posterior elución de las triazinas con 3 mL de acetona. Se evaluaron algunos aspectos sobre el pretratamiento de

la muestra tales como el almacenamiento de las triazinas en el cartucho de SPE y la filtración de la muestra. El tiempo máximo de almacenamiento en el cartucho a -18ºC es de tres semanas, y para la filtración se recomienda el uso de filtros de microfibra de vidrio porque con los filtros de membrana de nylon se obtienen peores recuperaciones para Simetrina y Terbutrina. El método es adecuado para la determinación de las triazinas estudiadas en aguas superficiales (río y mar). A pesar de que SPE es una técnica convencional de análisis, resulta de gran aplicabilidad en métodos de rutina ya que se comprobó que las triazinas son estables cuando se almacena el cartucho a -18ºC, lo que facilita el procesado de las muestras permitiendo realizar el análisis tiempo después de la toma de muestra.

Para poder llevar a cabo el análisis simultáneo de las triazinas y sus productos de degradación, empleando además ESI-MS/MS como sistema de detección, fue necesario realizar una serie de modificaciones del método; así el volumen de muestra se redujo a 10 mL dada la alta sensibilidad que presenta el detector de masas y se modificó el volumen de agua milli-Q utilizada en la etapa de lavado, para evitar pérdidas de los metabolitos más polares. Al no ser necesaria una etapa de concentración, se modificó también el tipo y volumen de eluyente, realizándose la elución con 2,5 mL de metanol. Aunque se han desarrollado métodos de SPE combinada con LC-MS/MS para la determinación de triazinas y alguno de sus productos de degradación en aguas de río, no se han realizado estudios en agua de mar.

El método de DLLME se desarrolló para la determinación de siete triazinas empleando DAD como sistema de detección y confirmación de los resultados por LC-ESI-MS/MS. En las condiciones optimizadas se emplean 25 mL de muestra con 300 µL de octanol como agente extractante y agitación a 1200 rpm durante 10 minutos. Es de interés destacar el pequeño volumen de disolvente orgánico utilizado y que el empleo de una etapa de agitación, en lugar del uso de un agente dispersante, simplifica el proceso. Sin embargo, presenta como desventaja que no se puede aplicar a la determinación de Prometrina y Terbutrina, ni a los productos de degradación.

Finalmente, como resultado de una estancia de investigación en el IAREN, se desarrolló un método de SPE acoplado en línea con UPLC-MS/MS para la determinación de triazinas y sus principales productos de degradación en agua de mar. En este dispositivo se emplea un sistema de doble columna OASIS® HLB insertado en el bucle de una válvula de inyección de seis vías. En las condiciones optimizadas, en la posición de "carga" se bombean 5mL de muestra a un flujo de 5 mL/min a través de una columna OASIS® HLB, previamente acondicionada con agua-metanol (95:5, v/v). A continuación la válvula conmuta a la posición de "inyección" y los analitos son eluidos por la fase móvil para obtener, finalmente, su separación en la columna

cromatográfica. Destacar que en este proceso la manipulación de la muestra es mínima, así como su contaminación o las posibles pérdidas de analitos. Además, al introducir en la columna toda la muestra de la que se van a extraer los analitos, permite la utilización de volúmenes más pequeños de disolvente que cuando la SPE se realiza fuera de línea. Por otro lado, empleando sólo 5 mL de muestra se alcanzan bajos límites de cuantificación que permiten la determinación de estos contaminantes en los niveles requeridos por la Directiva 2013/39/EU. Este hecho sumado a la rapidez del análisis (11 min) lo convierte en una posible herramienta para controlar la presencia de triazinas y sus productos de degradación a nivel de trazas en muestras de agua de mar en cumplimiento con las directivas de la UE. Es importante destacar que no se han realizado estudios empleando la técnica de SPE on-line-UPLC-MS/MS para la determinación de triazinas y sus productos de degradación en agua de mar.

En lo que respecta a matrices sólidas se ha optimizado metodología analítica basada en la dispersión de la matriz en fase sólida (MSPD) empleando DAD como sistema de detección para la determinación de las nueve triazinas objeto de estudio en sedimentos, algas, mariscos y pescados. Además, en el caso de los sedimentos, también se analizaron productos de degradación empleado MS/MS como sistema de detección. Debido a la diferente naturaleza y complejidad de las distintas matrices analizadas, para cada una de ellas se optimizó la cantidad de muestra empleada, y el tipo y cantidad de agentes dispersantes, coadsorbentes y eluyentes utilizados.

Es importante resaltar que aunque la técnica de MSPD ha sido ampliamente empleada en la extracción de residuos de diferentes plaguicidas en tejidos, tanto de origen vegetal como animal, en lo que respecta a herbicidas triazínicos son muy pocas las referencias que emplean este tipo de extracción. En lo que respecta a muestras sólidas medioambientales como suelos o sedimentos, las aplicaciones de esta técnica son muy escasas y recientes, y en lo que respecta a los herbicidas triazínicos, sólo se encuentran dos referencias que utilizan esta técnica de extracción.

Una vez optimizados los métodos, todos ellos fueron validados en términos de linealidad, sensibilidad, exactitud y precisión siguiendo los parámetros y criterios de validación de la Guía SANCO obteniéndose unos resultados altamente satisfactorios, cumpliendo todas las metodologías los requisitos exigidos por la Legislación vigente para cada tipo de matriz.

En el caso de algas marinas el método optimizado consiste en la trituración de 1 g de alga verde en un mortero empleando 2 g de C_8 como agente dispersante, seguida de transferencia a una columna que contiene los adsorbentes EnviCarbII-PSA (0,5 g/0,5 g). Una vez realizado un lavado de interferencias con 10 mL de hexano, la elución de las triazinas se lleva a cabo con 20 mL de acetato de etilo y 5 mL de acetonitrilo.

Finalmente, el método se aplicó al análisis de muestras de algas rojas y pardas proporcionando unos resultados satisfactorios en términos de recuperación y precisión, por lo que puede ser aplicado en programas de monitorización y control de distintos tipos de algas. Es importante señalar que los estudios de triazinas en algas son muy escasos, y que en ninguno de ellos se emplea la dispersión de la matriz en fase sólida como método de extracción.

En lo que respecta a mariscos, en las condiciones optimizadas, el método emplea 0,5 g de mejillón, 2 g de C_{18} como dispersante y ENVI-Carb-II/SAX/PSA (0,5 g/0,5 g/0,5 g) como coadsorbentes. A continuación, se llevan a cabo las etapas de lavado y elución siguiendo el mismo procedimiento que en el método optimizado para algas. En el caso de pescados, debido al mayor contenido lipídico de la trucha de acuicultura, fue necesario reducir la cantidad de muestra (0,2 g) para poder aplicar el método de manera satisfactoria. Resaltar que se ha mostrado por primera vez la aplicabilidad de este método de extracción en la determinación de las nueve triazinas en mariscos y pescados.

En cuanto a los sedimentos marinos, en las condiciones finales, se emplea 1 g de sedimento con 1 g de ENVI-Carb (como agente dispersante) y 20 mL de acetato de etilo como eluyente. Posteriormente este método se modificó con el objetivo de llevar a cabo la determinación simultánea de las triazinas y sus productos de degradación empleando además MS/MS como sistema de detección. Para ello, debido a la mayor polaridad de los productos de degradación, en la etapa de elución se emplearon 5 mL adicionales de acetonitrilo. Importante señalar que la única referencia en la bibliografía para la determinación de triazinas en sedimentos mediante MSPD, sólo incluye una triazina (Atrazina) en el estudio y ningún producto de degradación.

Finalmente los métodos se aplicaron al análisis de muestras de interés. Así el método SPE-HPLC-DAD se aplicó al análisis de cincuenta muestras de agua de mar recogidas en las rías de Arousa y Vigo, ambas ampliamente dedicadas a la pesca y acuicultura. Los métodos de DLLME-HPLC-DAD, MSPD-HPLC-DAD, SPE-LC-ESI-MS/MS y MSPD-LC-ESI-MS/MS se aplicaron al análisis de agua de mar y sedimentos recogidos en diez puntos susceptibles de contaminación por triazinas en la ría de A Coruña. El método en línea SPE-UPLC-MS/MS se aplicó al análisis de diez muestras de agua de mar recogidas en diez playas susceptibles de contaminación por triazinas en la costa de Matoshinos. En lo que respecta a biota, se analizaron diferentes muestras de algas y mejillones destinados a consumo humano procedentes de distintos mercados locales de A Coruña.

Aunque sólo en una muestra de sedimento se detectó la presencia de Terbutilazina, el análisis de estos compuestos es de gran interés de cara a evaluar los

riesgos para la salud humana, así como controlar la calidad del medio ambiente marino. Además, este estudio es de especial relevancia en Galicia, donde la industria de productos del mar representa una fuerte actividad económica, exportando sus productos a todo el mundo.

Por último, como fruto de la actividad docente desarrollada durante la realización de esta Tesis Doctoral, se ha diseñado un experimento de laboratorio para estudiantes del Grado de Química. Este proyecto consiste en la determinación de cuatro triazinas en agua de mar mediante SPE seguida de análisis mediante HPLC-UV. El principal objetivo del experimento es el aprendizaje de la instrumentación, así como de los principios implicados en el análisis cromatográfico. Además, permite a los estudiantes ganar experiencia en una serie de técnicas esenciales en un laboratorio de Química Analítica y familiarizarse con algunos principios de la Química Verde.

Os herbicidas triazínicos constitúen un dos grupos de herbicidas máis empregados a nivel mundial. Estes compostos son tóxicos e resistentes puidendo permanecer moitos anos no solo, na auga e nos organismos. Aínda con seren persistentes, pódense degradar dependendo das condicións químicas e biolóxicas do medio no que se atopan, dando lugar a metabolitos que tamén poden presentar efectos tóxicos. Como consecuencia, algún destes herbicidas pasaron a ser lexislados, emporiso outros herbicidas e os seus produtos de degradación aínda non se atopan regulamentados. Tras a súa aplicación, a través do ciclo hidrolóxico, estes compostos acadan o medio mariño, distribuíndose en tódolos seus compartimentos (auga, sedimentos, flora e fauna) en función das propiedades deste e da natureza das fases adsorbentes.

O medio mariño é un patrimonio moi valioso que debe ser protexido, conservado e rehabilitado para que os océanos e os mares estean limpos, sendo deste xeito sans e produtivos. En consecuencia, son necesarios máis estudos sobre o comportamento, efectos e presenza destes compostos neste medio. Por todo isto, requírense métodos analíticos que permitan a súa determinación a baixos niveis de concentración que sexan eficaces, precisos, sinxelos e rápidos. Nesta Tese desenvolveuse metodoloxía analítica para a determinación simultánea de nove herbicidas triazínicos (Ametrina, Atrazina, Cianazina, Prometrina, Propazina, Simazina, Simetrina, Terbutilazina e Terbutrina) e oito dos seus principais produtos de degradación (Desetil-Atrazina, Desetil-Desisopropil-Atrazina, Desetil-2-Hidroxi-Atrazina, Desetil-Terbutilazina, Desisopropil-Atrazina, Desisopropil-2-Hidroxi-Atrazina, 2-Hidroxi-Atrazina e 2-Hidroxi-Terbutilazina) en distintos compartimentos do ecosistema mariño. Cómpre destacar que na determinación destes compostos utilizáronse dende técnicas analíticas convencionais ata técnicas máis novas e sofisticadas, as cales foron empregadas por primeira vez ou para estes compostos ou para as matrices estudadas. Debido ás vantaxes que presentan e ós resultados satisfactorios obtidos, os métodos propostos melloran aqueles previamente desenvolvidos para este fin.

No que respecta ás técnicas instrumentais de análise, optimizáronse e validáronse tres métodos cromatográficos: cromatografía de líquidos de alta resolución con detección con rede de díodos (HPLC-DAD), cromatografía de líquidos con detección por espectrometría de masas en tándem (LC-MS/MS) e cromatografía de líquidos de ultra resolución con detección por espectrometría de masas en tándem (UPLC-MS/MS).

En primeiro lugar desenvolveuse un método de HPLC-DAD para a determinación das nove triazinas, empregando C_{18} como fase estacionaria e elución en gradiente con acetonitrilo-auga, lográndose unha boa separación e resolución para as

nove triazinas estudadas. A absorbancia mediuse no rango de 200-400 nm e a cuantificación realizouse a 222,7 nm cara a acadar a maior sensibilidade. É importante sinalar que non existen traballos na literatura nos que se logre unha adecuada resolución das nove triazinas empregando esta técnica; ademais, na meirande parte dos artigos atopados na bibliografía, Cianazina e Simazina presentan un total solapamento cando se emprega acetonitrilo na fase móbil.

A continuación púxose a punto un método LC-ESI-MS/MS para a determinación das nove triazinas e os seus oito principais produtos de degradación, empregando como modo de adquisición MRM (multiple reaction monitoring), debido á súa sensibilidade e selectividade, C_{18} como fase estacionaria e elución en gradiente con acetonitrilo-disolución acuosa ao 0,3% de ácido acético. A optimización das condicións de MS/MS incluíu a optimización do cono de voltaxe e a enerxía de colisión para cada pesticida, ao gallo de obter o ion precursor e os produtos correspondentes. É importante salientar que non se teñen atopado referencias nas que se realice a determinación simultánea dos dezasete compostos estudados.

Finalmente, canto ás técnicas instrumentais se refire, optimizouse un método de UPLC-MS/MS para a determinación das nove triazinas e os seus oito produtos de degradación, empregando C_{18} enlazada a partículas híbridas con pontes de etileno (BEH) como fase estacionaria e elución en gradiente con metanol-auga, contendo cada unha das fases móbiles acetato amónico 5 mM como modificador. O emprego de columnas curtas empaquetadas con pequeno tamaño de partícula presenta coma vantaxes, respecto á cromatografía de líquidos convencional, un menor tempo de análise así como de consumo de disolvente, maior eficiencia dos picos cromatográficos e melloras nos límites de detección. Por outra banda, é a primeira vez que se emprega esta técnica para a determinación simultánea dos dezasete compostos obxecto de estudo.

En relación aos métodos de extracción, optimizouse e validouse diferente metodoloxía analítica en auga de mar, sedimentos e biota (algas, mariscos e pescados). No caso de auga de mar desenvolvéronse tres procedementos analíticos: extracción en fase sólida fóra de liña (SPE off-line), extracción en fase sólida en liña (SPE on-line) e microextracción líquido-líquido dispersiva (DLLME). Para os tres métodos obtivéronse os Estándares Medioambientais de Calidade (EQS) da lexislación vixente no momento do estudo.

En primeiro lugar desenvolveuse un método de SPE off-line para a determinación das nove triazinas empregando DAD como sistema de detección. O método consiste na extracción e preconcentración de 50 mL de mostra de auga empregando cartuchos HLB, lavado de interferencias con 20 mL de auga Milli-Q e posterior elución das triazinas con 3 mL de acetona. Avaliáronse algúns aspectos sobre

o pretratamento da mostra tal e como o almacenamento das triazinas no cartucho de SPE e a filtración da mostra. O tempo máximo de almacenamento no cartucho a -18ºC é de tres semanas, e para a filtración recoméndase o uso de filtros de microfibra de vidro porque cos filtros de membrana de nylon obtéñense peores recuperacións para Simetrina e Terbutrina. O método é adecuado para a determinación das triazinas estudadas en augas superficiais (río e mar). Pese a que SPE é unha técnica convencional de análise, resulta de gran aplicabilidade en métodos de rutina xa que se comprobou que as triazinas son estables cando se almacena o cartucho a -18º C, o que facilita o procesado das mostras permitindo realizar a análise tempo despois da mostraxe.

Para poder levar a cabo a análise simultánea das triazinas e os seus produtos de degradación, empregando ademais ESI-MS/MS como sistema de detección, foi necesario realizar unha serie de modificacións do método; así o volume de mostra reduciuse a 10 mL debido á alta sensibilidade que presenta o detector de masas, e diminuíuse o volume de auga milli-Q empregada na etapa de lavado, para evitar perdas dos metabolitos máis polares. Ademais, ao non se precisar unha etapa de concentración, tamén se modificou o tipo e volume de eluínte realizándose a elución con 2,5 mL de metanol. Aínda que na bibliografía se poden atopar métodos de SPE combinada con LC-MS/MS para a determinación de triazinas e algún dos seus produtos de degradación en augas de río, non se teñen realizado estudos en auga de mar.

O método de DLLME desenvolveuse para a determinación de sete triazinas utilizando DAD como sistema de detección e confirmando os resultados obtidos por ESI-MS/MS. Nas condicións optimizadas empréganse 25 mL de mostra con 300 µL de octanol como axente extractante e axitación a 1200 rpm durante 10 minutos. É de interese resalzar o pequeno volume de disolvente orgánico utilizado e que o emprego dunha etapa de axitación, no canto do uso dun axente dispersante, simplifica o proceso. Porén, presenta como desventaxa que non se pode aplicar á determinación de Prometrina e Terbutrina, nin aos produtos de degradación.

Finalmente, como resultado dunha estadía de investigación no IAREN, desenvolveuse un método de SPE acoplada en liña con UPLC-MS/MS para a determinación de triazinas e os seus principais produtos de degradación en auga de mar. Neste dispositivo emprégase un sistema de doble columna OASIS® HLB insertado no bucle dunha válvula de inxección de seis vías. Nas condicións optimizadas, na posición de "carga" bombéanse 5 mL de mostra a un fluxo de 5 mL/min a través dunha columna OASIS® HLB, previamente acondicionada con auga:metanol (95:5, v/v). Seguidamente a válvula troca á posición de "inxección" e os analitos son eluidos pola fase móbil para obter, finalmente, a súa separación na columna cromatográfica. Destacar que neste proceso a manipulación da mostra é mínima, así como a súa contaminación ou as

posibles perdas de analitos. Ademais, ao introducir na columna toda a mostra da que se van extraer os analitos, permite a utilización de volumes máis pequenos de disolvente que cando a SPE se realiza fóra de liña. Por outra banda, empregando só 5 mL de mostra acádanse baixos límites de cuantificación que permiten a determinación destes contaminantes nos niveis requiridos pola Directiva 2013/39/EU. Este feito, engadido á rapidez da análise (11 min), convírteno nunha posible ferramenta para controlar a presenza de triazinas e os seus produtos de degradación a nivel de trazas en mostras de auga de mar no cumprimento coas directivas da UE. É importante salientar que é a primeira vez que se emprega a técnica de SPE on-line-UPLC-MS/MS para a determinación de triazinas e os seus produtos de degradación en auga de mar.

Respecto ás matrices sólidas, optimizouse metodoloxía analítica baseada na dispersión da matriz en fase sólida (MSPD) empregando DAD como sistema de detección para a determinación das nove triazinas obxecto de estudo en sedimentos, algas, mariscos e pescados. Ademais, no caso dos sedimentos, tamén se analizaron produtos de degradación empregando MS/MS como sistema de detección. Debido á diferente natureza e complexidade das distintas matrices analizadas, para cada unha delas optimizáronse a cantidade de mostra empregada, e os tipos e cantidades de axentes dispersantes, coadsorbentes e eluíntes utilizados.

Cómpre resaltar que aínda que a técnica de MSPD ten sido amplamente empregada na extracción de residuos de diferentes praguicidas en tecidos, tanto de orixe vexetal como animal, no que respecta aos herbicidas triazínicos son moi poucas as referencias que utilizan este tipo de extracción. No que atinxe a mostras sólidas medioambientais como solos ou sedimentos, as aplicacións desta técnica son moi escasas e recentes, e no referente aos herbicidas triazínicos, só se atopan dúas referencias que empregan esta técnica de extracción.

Unha vez optimizados os métodos, todos eles foron validados en termos de linealidade, sensibilidade, exactitude e precisión seguindo os parámetros e criterios de validación da Guía SANCO, obténdose uns resultados altamente satisfactorios, cumprindo tódalas metodoloxías propostas os requisitos esixidos pola Lexislación vixente para cada tipo de matriz no momento do estudo.

No caso das algas mariñas o método optimizado consiste na trituración de 1 g de alga verde nun morteiro empregando 2 g de C_8 como axente dispersante, seguida de transferencia a unha columna que contén os adsorbentes EnviCarbII-PSA (0,5 g/0,5 g). Unha vez realizado un lavado de interferencias con 10 mL de hexano, a elución das triazinas lévase a cabo con 20 mL de acetato de etilo e 5 mL de acetonitrilo. Finalmente, o método foi aplicado á análise de mostras de algas vermellas e pardas proporcionando uns resultados satisfactorios en termos de recuperación e precisión, polo que pode ser

aplicado en programas de monitorización e control de distintos tipos de algas. É importante sinalar que os estudos de triazinas en algas son moi escasos, e que en ningún deles se emprega a dispersión da matriz en fase sólida como método de extracción.

No que atinxe a mariscos, nas condicións optimizadas, o método emprega 0,5 g de mexillón, 2 g de C_{18} como dispersante e ENVI-Carb-II/SAX/PSA (0,5 g/0,5 g/0,5 g), como coadsorbentes. Deseguido, efectúanse as etapas de lavado e elución do mesmo xeito ca no método optimizado para algas. No caso de pescados, debido ao maior contido lipídico da troita de acuicultura, foi necesario reducir a cantidade de mostra (0,2 g) para poder aplicar o método satisfactoriamente. Subliñar que se mostrou por primeira vez a aplicabilidade deste método de extracción para a determinación das nove triazinas en mariscos e pescados.

Respecto aos sedimentos mariños, nas condicións finais, emprégase 1 g de sedimento con 1 g de ENVI-Carb (como axente dispersante) e 20 mL de acetato de etilo como eluínte. Posteriormente este método foi modificado co obxectivo de levar a cabo a determinación simultánea das triazinas e os seus produtos de degradación empregando ademais MS/MS como sistema de detección. A este fin, debido á maior polaridade dos produtos de degradación, empregáronse 5 mL adicionais de acetonitrilo na etapa de elución. Importante sinalar que a única referencia recollida na bibliografía para a determinación de triazinas en sedimentos mediante MSPD só inclúe unha triazina (Atrazina) no estudo e ningún produto de degradación.

Finalmente os métodos foron aplicados á análise de mostras de interese. Así o método SPE-HPLC-DAD aplicouse á análise de cincuenta mostras de auga de mar recollidas nas rías de Arousa e Vigo, ambas as dúas amplamente adicadas á pesca e á acuicultura. Os métodos de DLLME-HPLC-DAD, MSPD-HPLC-DAD, SPE-LC-ESI-MS/MS e MSPD-LC-ESI-MS/MS foron aplicados á análise de auga de mar e sedimentos recollidos en dez puntos susceptibles de contaminación por triazinas na ría de A Coruña. O método on-line SPE-UPLC-MS/MS aplicouse á análise de dez mostras de auga de mar recollidas en dez praias susceptibles de contaminación por triazinas na costa de Matosinhos. No que respecta á biota, analizáronse diferentes mostras de algas e mexillóns destinadas ao consumo humano, adquiridas en distintos mercados locais da cidade de A Coruña.

Aínda que só nunha mostra de sedimento se detectou a presenza de Terbutilazina, a análise destes compostos é de grande interese cara a avaliar os riscos para a saúde humana, así como controlar a calidade do medio ambiente mariño. Ademais, este estudo é de especial relevancia en Galicia, onde a industria dos produtos do mar representa unha forte actividade económica, exportando os seus produtos por todo o mundo.

Finalmente, como froito da actividade docente desenvolvida durante a realización desta Tese Doutoral, deseñouse un experimento de laboratorio para estudantes do Grao de Química. Este proxecto consiste na determinación de catro triazinas en auga de mar mediante SPE seguida de análise utilizando HPLC-UV. O principal obxectivo do experimento é a aprendizaxe da instrumentación, así como dos principios implicados na análise cromatográfica. Ademais, permite aos estudantes gañar experiencia nunha serie de técnicas esenciais nun laboratorio de Química Analítica e familiarizarse con algúns dos principios da Química Verde.

Triazines are a group of herbicides worldwide used. These compounds are toxic and resistant and can remain many years in soil, water and organisms. Despite being persistent, they can be transformed depending on the biological and chemical conditions of the medium where are present. Knowledge about the degradation products is of great interest because they may have toxic effects. Some of these herbicides have been legislated while others and their degradation products are still not regulated. After their application, fluxes of these compounds can reach the marine environment through hydrological cycle. Once there, they are distributed in all compartments (water, sediments and biota) depending on the properties of the marine ecosystem and the nature of the adsorbent phases.

The marine environment is a valuable asset that has to be protected, conserved and rehabilitated in order to keep clean oceans and seas being healthy and productive. Consequently, further studies about the behaviour, effects and occurrence of these compounds in the marine environment are required. For this purpose, analytical methods that allow the determination of these compounds at low concentration levels are necessary, which must be also accurate, effective, simple and fast. In this Thesis, analytical methodology for the simultaneous determination of nine triazines (Ametryn, Atrazine, Cyanazine, Prometryn, Propazine, Simazine, Simetryn, Terbuthylazine and Terbutryn) and eight of their main degradation products (Desethyl-Atrazine, Desethyl-Desisopropyl-Atrazine, Desethyl-2-Hydroxy-Atrazine, Desethyl-Terbuthylazine, Desisopropyl-Atrazine, Desisopropyl-2-Hydroxy-Atrazine, 2-Hydroxy-Atrazine and 2-Hidroxy-Terbuthylazine) in different compartments of the marine ecosystem has been developed. It should be noted that from conventional to more sophisticated and novel analytical techniques have been employed for the determination of the compounds. These techniques have been used for the first time either for the analysis of these compounds or for the matrices studied. Due to their advantages and satisfactory results obtained, the proposed methods improve the previous ones developed with the same purpose.

Concerning analytical techniques, three chromatographic methods were optimized and validated: high performance liquid chromatography with diode array detection (HPLC-DAD), liquid chromatography with tandem mass spectrometry detection (LC-MS/MS) and ultra performance liquid chromatography with tandem mass spectrometry detection (UPLC-MS/MS).

At the beginning, a HPLC-DAD method was developed for the determination of nine triazines, employing C_{18} as stationary phase and gradient elution with acetonitrile-water. An adequate separation for all the compounds was achieved. The absorbance was measured continuously in the 200-400 nm range and peaks areas quantification was

carried out at 222,7 nm in order to achieve maximum sensitivity. It is important to note that there are no references in the literature which achieve a good resolution of the nine triazines; in addition, in most of the articles, Cianazine and Simazine remain total overlapped when acetonitrile is employed in the mobile phase.

Subsequently a LC-ESI-MS/MS method for the determination of nine triazines and their eight main degradation products using MRM (multiple reaction monitoring) as acquisition mode, C_{18} as stationary phase and gradient elution employing acetonitrile-0,3% acetic acid in water was developed. The optimization of MS/MS conditions included the optimization of cone voltage and collision energy for each pesticide in order to obtain the precursor ion and the corresponding products. It is noteworthy that no references have been found devoted to the simultaneous determination of these seventeen compounds.

Finally, as far as instrumental techniques is concern, an UPLC-MS/MS method for the determination of nine triazines and their eight degradation products using C_{18} bonded to ethylene-bridged hybrid (BEH) particles as stationary phase and gradient elution employing methanol-water with 5 mM ammonium acetate as modifier in both mobile phases was optimized. Respect to conventional liquid chromatography, the use of short packed columns with small particle size has advantages such as significantly shorten the analysis time as well as solvent consumption, higher efficiency of the chromatographic peaks and improvements in the detection limits. On the other hand, it is the first time that this technique is used for the simultaneous determination of these seventeen compounds.

Regarding extraction methodologies, several analytical methods have been optimized and validated for the determination of the target compounds in seawater, sediment and biota (seaweed, fish and shellfish). With respect to seawater, three analytical procedures have been developed: solid phase extraction off-line (SPE off-line), solid phase extraction on-line (SPE on-line) and dispersive liquid liquid microextraction (DLLME). The three methods achieved the Environmental Quality Standards (EQS) established by Legislation in force at the moment of the study.

In the first study, a SPE off-line method for the determination of nine triazine herbicides employing DAD as detection system was developed. The method consists on the extraction and preconcentration of 50 mL of water sample through HLB cartridges, rinsing with 20 mL of Milli-Q water and elution with 3 mL of acetone. Furthermore several aspects concerning sample pre-treatment, such as the sample filtration step and stability of herbicides on the cartridge, were also evaluated. It could be concluded that the integrity of the analytes at -18ºC during three weeks was not affected. Regarding filtration step, glass microfiber filters are recommended because an important decrease

on recoveries of Simetryn and Terbutryn were observed when nylon membrane filters were employed. In addition, the proposed method is useful to determine triazines in surface water (river and sea). It is noteworthy that although SPE is a conventional technique, it is a good alternative since the use of cartridges allows the storage of the triazines until analysis, avoiding the problem associated with the maintaining herbicides integrity in aqueous solution when long periods are required before analysis.

Subsequently, the SPE method was modified with the aim of analysing simultaneously triazines and their main degradation products employing, in addition, the detector MS/MS. For this purpose, sample volume was reduced to 10 mL due to the high sensitivity of mass detector, and milli-Q volume used for the washing step was also reduced to avoid losses of the more polar metabolites. Furthermore, due to a concentration step was not necessary, elution was carried out with 2,5 mL of methanol. It is noteworthy that methods based on solid-phase extraction combined with LC-MS/MS have been used to measure triazines and some degradation products in river waters; however, there are not studies in seawater.

Then a DLLME method was developed for the determination of seven triazines employing DAD detection and confirmation of the results by ESI-MS/MS detection. Under optimized conditions, 25 mL of seawater sample were extracted with 300 µL of octanol as extractant solvent by shaken at 1200 rpm for 10 minutes. It is important to note that the method employs small volume of organic solvent and that the use of an agitation step instead of a dispersive solvent, simplifies the experimental procedure. However, this method has the disadvantage that it cannot be applied to the determination of Prometryn and Terbutryn, neither to degradation products.

Finally, as result of a research stay on the IAREN, an on-line SPE method coupled with UPLC-MS/MS was developed for the determination of triazines and their degradation products in seawater. The extraction of seawater was performed by loading 5mL of the solution at 5 mL/min through an OASIS® HLB on-line SPE cartridge previously conditioned with water-methanol (95:5, v/v). The trap cartridge was fitted into “load” position of a valve switching Rheodyne port. After sample loading, the valve was switched to “injection” position being the analytes eluted to the LC column with the chromatographic mobile phase. On-line SPE devices provide advantages, including high sample throughput, minimal solvent utilization, fast sample preparation and small sample volume. This method enables the determination of these pollutants at the levels required by European Union legislation; consequently, it can be an important tool to control the presence of triazines and their degradation products in seawater samples. To best of our knowledge, no studies using on-line SPE-UPLC-MS/MS have been done to determine the target compounds in seawater.

As regards to solid matrices, analytical methodology based on Matrix Solid Phase Dispersion using DAD as detection system has been optimized for the determination of the nine triazines in sediments, seaweeds, fish and shellfish. Furthermore, in the case of sediments, degradation products were also analyzed employing MS/MS as detection system. Because of the different nature and complexity of the several matrices, it was necessary to optimize for each one the sample amount and the type and amount of dispersing agents, co-sorbents and elution solvents.

MSPD technique has been widely used for the extraction of different pesticide residues from biological tissues, with vegetal and/or animal origin; however it is worthy noting that references for the determination of triazines by MSPD are still scarce and furthermore, in most cases, few triazines are included in these studies. Regarding environmental matrices, such as soils or sediments, studies of triazines employing this technique are limited and recent, and to the best of our knowledge there are only two references devoted to the determination of triazines employing this extraction technique.

Once the analytical methods were optimized, all of them were validated in terms of linearity, sensitivity, precision and accuracy according to validation parameters and criteria from SANCO Guidelines. The obtained results were highly satisfactory for all developed methodologies, indicating that all methods meet the requirements stipulated in compliance with EU legislation.

In the case of seaweed, under optimized conditions, the procedure consists on blending 1 g of green seaweed in a mortar employing C_8 as dispersing agent (2 g) and subsequent transferring into a SPE column cartridge containing Envi-CarbII/PSA (0,5 g/0,5 g) as clean-up co-sorbent. Then the dispersed sample was washed with 10 mL of hexane and triazines were eluted with 20 mL of ethyl acetate and 5 mL of acetonitrile. Finally, the reliability of method was evaluated in terms of recovery by spiking a red seaweed and a brown one. The obtained results demonstrated that the method achieved satisfactory recoveries for all compounds, which indicates that this procedure could be established as a suitable methodology for routine analysis to screen and monitor triazines in different types of seaweeds in compliance with EU legislation. It is worthy to note that studies of triazines in seaweeds are very scarce and recent, and no studies using MSPD have been done to extract triazines from seaweeds.

With regards to shellfish, under final working conditions, 0,5 g of mussel was homogenized with 2 g of C_{18} and then the final mixture was transferred into a SPE column containing a triple sorbent layer of Envi-CarbII/SAX/PSA (0,5 g/0,5 g/0,5 g). Once packed MSPD columns, the washing and elution steps were performed following the procedure previously described for seaweed samples. In relation to fish, because of the higher lipid content in aquaculture trout than in mussel, it was necessary to reduce

the sample amount to 0,2 g. It is important to highlight that the suitability of a procedure based on MSPD for the extraction of these chemicals residues from fish and shellfish has been demonstrated for the first time.

Concerning to sediments, under optimized conditions, 1 g of sediment sample was blended with 1 g of ENVI-Carb in a glass mortar with a pestle for 5 min. The final mixture was transferred into a glass syringe and once packed, elution was performed with 20 mL of ethyl acetate. Then, the MSPD method was modified with the aim of analysing simultaneously triazines and their main degradation products employing, in addition, the detector MS/MS. For this purpose, due to the higher polarity of the degradation products, the eluent was slightly modified adding 5 mL of acetonitrile to the eluent of the initial method. To the best of our knowledge there is only one reference in the literature devoted to the determination of triazines by MSPD in sediments; furthermore, this study only includes one triazine (Atrazine) and none degradation product.

Finally the developed methods were applied to the analysis of samples. The SPE-HPLC-DAD method was applied to analyze fifty seawater samples collected in the estuaries of Arousa and Vigo, both areas widely dedicated to shellfishing and fishing. The DLLME-HPLC-DAD, MSPD-HPLC-DAD, SPE-LC-ESI-MS/MS and MSPD-LC-ESI-MS/MS were applied to the analysis of seawater and sediments samples collected from ten points susceptible to contamination by triazines from estuary of a Coruña. The on-line SPE-UPLC-MS/MS method was applied to the analysis of triazines and their degradation products in ten seawater samples collected from ten beaches susceptible to contamination by triazines in the seashore of Matosinhos. Regarding biota samples, the MSPD-HPLC-DAD methods were applied to different edible samples of seaweeds and mussels purchased from local markets of A Coruña.

Although only one sample of sediment contained detectable amount of Terbuthylazine, the analysis of these compounds is of great interest in order to evaluate risks for human health and also to control the quality of the marine environment. Furthermore, this study is of a special relevance in Galicia, where the seafood industry is very important, exporting its products around the world.

Finally, as a result of the teaching activity carried out during the period of this Thesis, a laboratory experiment for undergraduate students of Chemistry Degree has been designed. This project is focused on the determination of four triazines in seawater samples using SPE followed by HPLC-UV. The main pedagogical goal of this experiment is the learning both the use of the instrument and the principles involved on the chromatographic analysis. Furthermore, it permits the students to gain experience on

a number of essential techniques in the laboratory of Analytical Chemistry and also to get familiarity with some of the principles of Green Chemistry.

ANEXOS

ANEXO I

PUBLICACIONES

ANEXO I. PUBLICACIONES

ANEXO I-I

Application of a Developed Method for the Extraction of Triazines in Surface Waters and Storage Prior to Analysis to Seawaters of Galicia (Northwest Spain). *The Scientific World Journal* (2013). http://dx.doi.org/10.1155/2013/536369.

Hindawi Publishing Corporation
The Scientific World Journal
Volume 2013, Article ID 536369, 6 pages
http://dx.doi.org/10.1155/2013/536369

Research Article

Application of a Developed Method for the Extraction of Triazines in Surface Waters and Storage Prior to Analysis to Seawaters of Galicia (Northwest Spain)

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Received 28 August 2013; Accepted 17 September 2013

Academic Editors: H. A. Alegria and R. A. Smith

A simple method based on solid-phase extraction combined with liquid chromatography for simultaneous determination of nine triazine herbicides (ametryn, atrazine, cyanazine, prometryn, propazine, simazine, simetryn, terbuthylazine, and terbutryn) in surface water samples was developed and validated. Under optimized conditions, 50 mL of water sample was pumped through the Oasis HLB cartridge, and triazines were eluted with 3 mL acetone. Finally the extract was concentrated to dryness, reconstituted with 1 mL methanol : water (1 : 1) and injected into the HPLC-DAD system. The stability of the herbicides on the cartridges at −18 and 4°C was also evaluated, and the recoveries obtained after three weeks of storage were satisfactory for all compounds. The analytical features of the proposed method were satisfactory: repeatability and intermediate precision were <10% and recoveries in spiked river water and seawater samples were higher than 93% for all compounds studied. Limits of quantification (varied from 0.46 to 0.98 $\mu g\,L^{-1}$) were adequately allowing the determination of these compounds at the levels requested by the 2008/105/EC Directive. Finally, this method was applied to the analysis of 50 seawater samples from Galicia (northwest Spain).

1. Introduction

Triazines are recognised herbicides which have been broadly used in agriculture over the recent decades. The surface water receives fluxes of these compounds mainly of agricultural origin, due to their widespread use in this field [1]. Their high persistence and toxicity have required rigorous control of environmental contamination. Therefore, the presence of pesticides in surface waters is regulated by the European Directive 2008/105/EC that establishes a maximum permitted concentration of 2 and 4 $\mu g\,L^{-1}$ for atrazine and simazine, respectively [2]. It is important to take into account that atrazine and simazine have been included in the list of "priority hazardous substances" in Decision 2455/2001/EC [3] that amends the Directive 2000/60/EC [4], and atrazine, ametryn, prometryn, terbutryn, simazine, and propazine are considered as a group to be endocrine-disrupting chemicals by the US Environmental Protection Agency [5]. Thus, sensitive methods for determining the low concentrations of triazine herbicides in environmental samples are required.

Different chromatographic techniques have been used to determine triazines. Gas chromatography coupled to mass spectrometry has been widely employed [6–11], and the use of liquid chromatography with different detectors such as ultraviolet [12–16], diode array [17, 18], or mass spectrometry [19–25] has also been reported.

An extraction procedure to preconcentrate the analytes and remove possible interferences is mandatory to achieve the required levels. For this purpose, solid-phase extraction (SPE) is the preconcentration technique most commonly used for the determination of triazines in water samples [7–11, 17, 19–23, 25]. Recently, microextraction techniques have become important procedures in environmental analysis. Thus, some microextraction methods such as solid-phase microextraction (SPME) [26], stir bar sorptive extraction (SBSE) [6], liquid phase microextraction (LPME) [24],

liquid-liquid-solid microextraction (LLSME) [13], and dispersive liquid-liquid microextraction (DLLME) [14, 18, 27] have been applied for extraction and preconcentration of triazines in water as alternative to the SPE techniques. However, some of these new techniques have drawbacks like low sensitivity for the triazines studied [26], poor recoveries [14, 18], and in many cases they are very laborious [13, 24].

The aim of the current study was to develop a simple, sensitive, and low-cost method based on SPE for the extraction of nine triazines from surface water samples and the storage of the compounds until their determination by HPLC-DAD. Samples of river water and seawater were chosen to illustrate the reliability of the method. Finally, the method was applied to analyze 50 samples of seawater from two areas dedicated to shellfishing and fishing.

2. Experimental

2.1. Site Location and Sampling. Fifty seawater samples from Galicia (northwest Spain) were collected at two different locations (zones 1 and 2) from April to June 2011. The health of both estuaries is a priority for the government of the region, because they are engaged in shellfish and fishing. In zone 1, (estuary of Arousa Island) 37 sites were selected for sampling and in zone 2, (estuary of Vigo) 13 sites were selected. The sampling locations and their designations are shown in Figure 1. At each sampling location three samples were collected.

Samples were collected in amber glass bottles and transported to the laboratory under cooled conditions (4°C). Upon reception, samples were filtered through 0.6 μm glass fibre filters to eliminate suspended solid matter and the solid-phase extraction was carried out. The cartridges were stored at −18°C in the dark until analysis.

2.2. Chemical and Materials. All herbicides analytical standards were supplied by Sigma-Aldrich (Inc. St. Louis, MO, USA). The individual stock standard solutions of 1000 mg L^{-1} were prepared in methanol by exact weighing of high-purity substances and stored at–18°C in the dark. Then a mixture of all the compounds was prepared in methanol containing 10 mg L^{-1} each individual triazine and stored at −18°C. All working solutions were daily prepared by appropriate dilution of the 10 mg L^{-1} standard solutions with methanol:water (1 : 1, v/v).

Acetonitrile was purchased from Panreac (Barcelona, Spain) and methanol and acetone from Romil (Cambridge, UK). All chemicals were of HPLC grade. Ultra-pure Milli-Q water was obtained using a Millipore Milli-Q system (Millipore, Bedford, MA, USA).

Reverse phase polymeric cartridges Oasis HLB (6 mL, 200 mg) were supplied by Waters (Milford, MA, USA). Glass fibre MN GF-6 filters (0.6 μm pore size) were purchased from Macherey Nagel (Düren, Germany) and 0.20 μm pore-size nylon membrane filters from Millipore (Bedford, MA, USA).

A visiprep vacuum system from Supelco (Bellefonte, PA, USA), a rotary evaporator (Büchi, Labortechnic AG, Flawil, Switzerland), and an ultrasonic bath (Branson 3200, Energieweg, The Netherlands) were used.

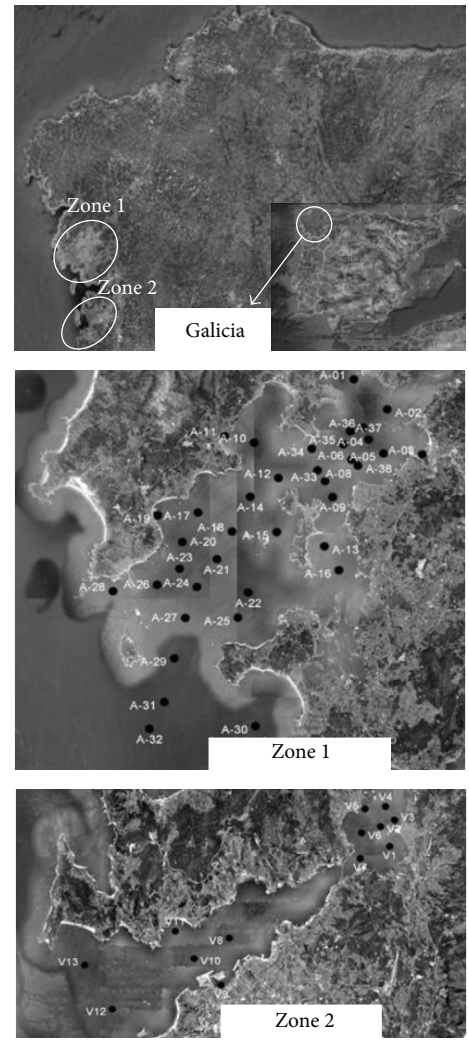

Figure 1: Map of sampling sites location at Galicia (NW Spain).

Uncontaminated river and seawater samples collected from a brook and Riazor beach in the city of A Coruña (Galicia, NW Spain) were used for the validation of the method.

2.3. Instrumental Analysis. Chromatographic analyses were performed using a high performance liquid chromatography-diode array detector (HPLC-DAD). The system consisted of a 2695 pump with a 996 Diode Array Detector from Waters (Milford, MA, USA). The column was a stainless steel column (150 mm × 4.6 mm ID, particle size 5 μm) packed with Hypersil GOLD C_{18} chemical bonded phase from Thermo Scientific (Austin, TX, USA).

The analysis was carried out using the following gradient elution: acetonitrile initial percentage of 30% (8 min) increased linearly to 40% in 5 min and increased to 50% in 5 min, after which the percentage was returned to the initial conditions in 9 min. The flow rate was 1 mL min^{-1}, the oven temperature was set at 25°C, and 20 μL of sample volume was used.

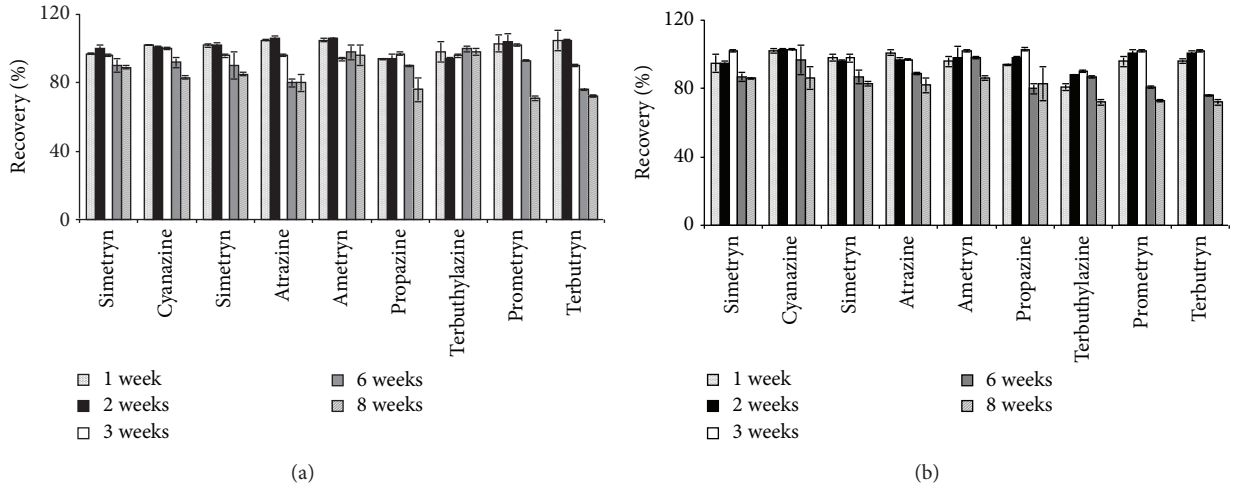

Figure 2: Effect of storage conditions on the recovery of the herbicides. (a) Temperature −18°C, (b) temperature 4°C.

The absorbance was measured continuously in the 200–400 nm range, and peaks areas quantification were carried out at 222.7 nm in order to achieve maximum sensitivity.

All triazine herbicides were identified initially by retention time and then by applying spectral contrast techniques (incorporated in Millenium^{32} software) homogeneity of the spectral peak was confirmed. Finally, a spectral identification was carried out contrasting the spectrum with a standard library created in wavelength interval of 200–400 nm.

2.4. Solid-Phase Extraction Procedure. The extraction was performed as follows: the cartridge was conditioned by washing it with 10 mL methanol and 10 mL Milli-Q water. Water sample (50 mL) was pumped through the cartridge at a flow rate of 10 mL min^{-1} and then the cartridge was washed with 20 mL Milli-Q water. Once the retention step had been completed, the cartridge was partially dried under a vacuum system for 5 min and then it was totally dried using a nitrogen stream for 30 min. The elution of retained compounds was done with 3 mL of acetone, and the organic extract was brought to complete dryness under a combination of rotary evaporator at 40°C and a gentle nitrogen stream. Finally the sample was reconstituted in methanol/water (1 : 1, v/v) to a final volume of 1 mL and injected into the HPLC.

3. Results and Discussion

3.1. SPE Method Optimization. The solid-phase extraction procedure was based on a previous method for drinking water developed by the same authors [28]. To optimize the method for surface waters, a filtration step prior to the SPE was studied. For this purpose, two different filters were assayed (glass microfiber filters and nylon membrane filters). A volume of 50 mL of seawater sample was spiked at a concentration level of 2 μg L^{-1} (the lowest legislation level). The spiked sample was mixed in an ultrasonic bath for 5 min to ensure efficient distribution of the herbicides, and it was allowed to equilibrate for 5 min prior to extraction, and then it was filtered. Isolation and determination of the compounds from the spiked samples were performed as described above.

Results showed an important decrease on the recovery for simetryn (68%) and terbutryn (50%) when nylon membrane filter was employed (recoveries between 82 and 101% were obtained for the other seven herbicides). However, values of recovery were satisfactory for all compounds when glass microfiber filter was used.

3.2. Stability of Herbicides on the Cartridges. Due to that herbicides may be degraded during storage of the sample at 4°C by processes such as hydrolysis or microbial decomposition, the fact of being able to perform the storage of the cartridges after the solid-phase extraction for extend time periods is extremely useful. Thus, the extraction can be performed on the day of sampling or even at the time of sampling for further transport to the laboratory. For this reason, the stability of the herbicides loaded on Oasis HLB cartridges was investigated under different storage conditions. Several cartridges were loaded with 50 mL of seawater spiked at 2 μg L^{-1} with a standard mixture of the nine herbicides and stored at 4 and −18°C. Elution of triazines was carried out after 1, 2, 3, 6, and 8 weeks of storage employing three cartridges for each time and temperature studied. Before elution, cartridges kept at −18°C and 4°C were defrosted at room temperature for 2 and 1 h, respectively. The organic extracts obtained were reconstituted and analyzed.

The effect on the recovery of the herbicides was evaluated. The results are shown in Figure 2. As can be seen (Figure 2(a)), triazines showed a high stability at −18°C being the recoveries quantitative after 6 weeks of storage for all the compounds (recovery values higher than 90% and RSD < 10%), except atrazine and terbutryn in which case recovery considerably decreased after 3 weeks (80 and 76%, resp., after 6 weeks of storage). However, the recovery of the compounds was only quantitative up to 3 weeks at 4°C, and it was decreasing gradually for higher storage periods of time, mainly for prometryn, propazine,

Table 1: Analytical characteristics of the SPE-HPLC method.

Compound	LOD ($\mu g\,L^{-1}$)	LOQ ($\mu g\,L^{-1}$)	Determination coefficient (r^2)	Repeatability[a] RSD (%)	Reproducibility[a] RSD (%)
Simazine	0.28	0.84	0.9962	4.3	10.0
Cyanazine	0.33	0.98	0.9910	4.4	4.8
Simetryn	0.23	0.70	0.9955	5.7	6.5
Atrazine	0.15	0.46	0.9981	5.5	7.6
Ametryn	0.28	0.85	0.9915	5.2	5.2
Propazine	0.18	0.56	0.9978	4.5	8.6
Terbuthylazine	0.26	0.80	0.9948	7.6	8.3
Prometryn	0.16	0.50	0.9982	1.4	2.4
Terbutryn	0.26	0.80	0.9966	3.1	5.1

[a] $n = 8$ and $n = 5$ for repeatability and reproducibility respectively ($2\,\mu g\,L^{-1}$).

and terbutryn (Figure 2(b)). On the other hand, a major variability on the recoveries was observed at 4°C. Therefore, it can be concluded that the most reliable method for storing the herbicides on the cartridge is keep them at –18°C during 3 weeks because the integrity of the analytes is not affected.

3.3. Method Validation. The analytical characteristics of the SPE-HPLC method were evaluated using a 50 mL of uncontaminated seawater sample spiked with a standard mixture of the compounds. The linearity was studied at 6 concentration levels (0.5, 1, 1.5, 2, 2.5, and 3 $\mu g\,L^{-1}$). As can be seen in Table 1, determination coefficients (r^2) were higher than 0.991 for all the herbicides at concentrations within the interval tested.

The limits of detection (LODs) were determined as $3 * S_{y/x}/b$ and the limits of quantification (LOQs) as $10 * S_{y/x}/b$, where $S_{y/x}$ is the residual standard deviation and b is the slope of the calibration curves. As can be seen in Table 1, the detection and quantification limits between 0.15–0.33 and 0.46–0.98 $\mu g\,L^{-1}$, respectively, were adequate, being the LOQs much lower than parametric value requested by the legislation for surface water [2].

Repeatability and intermediate precision were evaluated at $2\,\mu g\,L^{-1}$. The repeatability was calculated as within-day RSD of peak areas using eight replicates analyzed in the same day and by the same analyst. In the case of intermediate precision, five replicates were analyzed in consecutive days and by the same analyst and it was calculated as between-day RSD of peak areas. As can be seen in Table 1, the results obtained were satisfactory with RSD values below 10% for all compounds in both cases.

The accuracy (expressed as percent recovery) of the method was studied using seawater sample spiked with $2\,\mu g\,L^{-1}$ of a standard mixture of the compounds. The recoveries obtained for five replicates ($n = 5$) of the sample spiked with the triazine herbicides are presented in Table 2. The results demonstrated that the method achieved satisfactory recoveries in the range of 93%–106%, with associate standard deviations below 9% for all compounds.

Furthermore, a river water sample was also used to evaluate the reliability of the SPE-HPLC method. For this purpose, five replicates of 50 mL of sample spiked at $2\,\mu g\,L^{-1}$ with a standard mixture of the compounds were subjected to the optimized method and analytical recoveries were evaluated. The results obtained (see Table 2) have been shown to be satisfactory with recoveries in the range of 95%–104%, with RSD below 7% for all compounds.

Table 2: Study of analytical recovery in river and sea waters ($2\,\mu g\,L^{-1}$, $n = 5$).

Compound	Analytical recovery (%) ± RSD (%)	
	River water	Seawater
Simazine	100 ± 7.0	100 ± 7.8
Cyanazine	100 ± 6.1	106 ± 8.8
Simetryn	100 ± 3.1	103 ± 9.0
Atrazine	96 ± 5.3	93 ± 8.9
Ametryn	100 ± 5.1	102 ± 7.3
Propazine	99 ± 5.4	103 ± 8.7
Terbuthylazine	104 ± 2.5	99 ± 8.4
Prometryn	96 ± 6.6	106 ± 6.4
Terbutryn	95 ± 4.1	99 ± 2.3

Therefore, it can be concluded that the proposed method is useful to determine triazines in surface water samples. As an example, chromatograms corresponding to seawater (Figure 3(a)) and seawater sample spiked at a concentration level of $2\,\mu g\,L^{-1}$ (Figure 3(b)) are presented in Figure 3.

3.4. Application. Finally, the method was applied to the analysis of 50 seawater samples from Galicia (NW Spain). Although the herbicides under study have not been detected in the samples analyzed, studies to generate information related to their levels in areas where seafood is growing (mussels, crabs, oysters,...) are of great economic and environmental interest.

4. Conclusion

The proposed method provides a simple and inexpensive way for simultaneous determination of nine triazine herbicides in surface waters. Furthermore, it uses small volume of organic

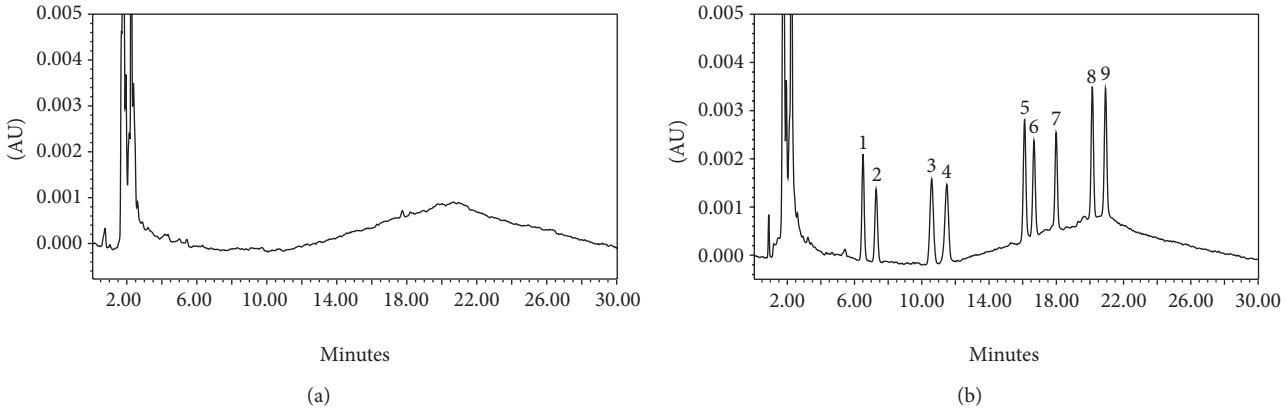

FIGURE 3: Chromatograms obtained after solid-phase extraction. (a) seawater sample, (b) seawater sample spiked at 2 μg L^{-1}. Target compounds are numbered as follows: (1) simazine, (2) cyanazine, (3) simetryn, (4) atrazine, (5) ametryn, (6) propazine, (7) terbuthylazine, (8) prometryn, and (9) terbutryn.

solvents in agreement with the principles of Green Chemistry. The stability of the herbicides on the cartridge stored at −18°C is of great interest and usefulness since the use of the cartridges allows the storage of the triazines until analysis, avoiding the problem associated with the maintaining herbicides integrity in aqueous solution when long periods of storage are required before analysis. This fact makes the solid-phase extraction procedure developed as a promising alternative to conventional water sampling for triazines analysis. However, further work should be necessary to optimize the extraction methodology "in situ" during sampling.

The method was successfully applied for river and seawater samples, and satisfactory precision, accuracy, and sensitivity were obtained. Using 50 mL of seawater sample, the LOQs obtained were lower than the parametric value requested by the legislation [2].

The method was applied to the analysis of 50 seawater samples from Galicia (NW Spain). Although the triazines under study have not been detected in the samples analyzed, the monitoring of their levels in marine ecosystems is of great economic and environmental importance. It is important to take into account that measurements of known quality represent the foundation of the water quality evaluation system and the basis for decisions to be taken to achieve the Marine Strategy Framework Directive and environmental objectives at the end of 2015 [29].

Finally, it is noteworthy that methods based on solid-phase extraction combined with liquid chromatography have been commonly used to measure triazines in drinking and ground waters; however, there are not studies in seawater.

Acknowledgments

The authors acknowledge the financial support of the "Ministerio de Educación y Ciencia" (Project CTQ2007-63949/BQU) and the "Xunta de Galicia" (Project 09MDS038103PR). N. Rodríguez-González also thanks the "Xunta de Galicia" for the concession of her award under the Program "Campus del mar".

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ANEXO I-II

An environmentally friendly method for determination of triazine herbicides in estuarine seawater samples by dispersive liquid-liquid microextraction.

Environmental Science and Pollution Research (2015) 22, 618-626.

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Environ Sci Pollut Res (2015) 22:618–626
DOI 10.1007/s11356-014-3383-9

RESEARCH ARTICLE

An environmentally friendly method for the determination of triazine herbicides in estuarine seawater samples by dispersive liquid–liquid microextraction

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Received: 25 February 2014 / Accepted: 23 July 2014 / Published online: 7 August 2014

Abstract A fast, simple, sensitive and green chemistry method using dispersive liquid–liquid microextraction (DLLME) for the simultaneous determination of seven triazine herbicides (ametryn, atrazine, cyanazine, propazine, simazine, simetryn and terbuthylazine) in estuarine seawater samples has been developed. DLLME was carried out using a small volume of seawater (25 mL) and 300 μL of 1-octanol. Herbicide concentrations were determined by liquid chromatography-diode array detection, and results were confirmed by liquid chromatography-electrospray ionisation tandem spectrometry analysis. The analytical features of the proposed method were satisfactory with repeatability < ±5 % and intermediate precision < ±10 %, and recoveries ranged from 81–102 % for all compounds. All the triazines exhibited linear matrix calibration curves with coefficients of determination >0.999 for all the analytes except for simazine (0.9975). Limits of quantification ranged between 0.19 and 1.12 μg L^{-1}. The method was applied to the analysis of seawater samples from ten points susceptible to contamination by triazines from estuary of A Coruña (Galicia, NW of Spain). The levels of the seven triazines were below the LODs in the analysed samples. Use of proposed method will allow for monitoring of triazines at levels below the regulatory limits set by the European Directive 2008/105/EC of 2 and 4 μg L^{-1} for atrazine and simazine, respectively.

Responsible editor: Laura McConnell

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Keywords Triazine herbicides · Seawater samples · Dispersive liquid–liquid microextraction · HPLC-DAD · LC-ESI-MS/MS

Introduction

Triazines are well-known herbicides that are worldwide applied to soil for the control of weeds in many agricultural crops, as well as railways, roadside and golf courses. The marine environment receives fluxes of these compounds mainly of agricultural origin. These compounds are highly resistant and can survive many years in soil, water and organisms. Therefore, they are considered an important class of chemical pollutants. Atrazine and simazine are included in the group of endocrine-disrupting compounds by the US Environmental Protection Agency (2009). Furthermore, the presence of pesticides in surface waters is regulated by the European Directive 2008/105/EC that establishes a maximum permitted concentration of 2 and 4 μg L^{-1} for atrazine and simazine, respectively (Council of the European Communities 2008a). It is important to take into account that atrazine and simazine have been included in the list of "priority hazardous substances" in Decision 2455/2001/EC (Council of the European Communities 2001) that amends the Water Framework Directive 2000/60/EC (Council of the European Communities 2000). In order to support the implementation of the Directive 2008/105/EC, fast, simple and sensitive analytical methods are required.

Different chromatographic techniques have been used to determine triazines. Gas chromatography coupled to mass spectrometry has been widely employed (Sanchez-Ortega et al. 2009; Bagheri et al. 2010; Djozan et al. 2010; Katsumata et al. 2010; Matamoros et al. 2010; Navarro et al. 2010; Portoles et al. 2011; Bagheri et al. 2012), and the use of liquid

chromatography with different detectors such as ultraviolet (Hu et al. 2009a, b; Zhou et al. 2009; Beale et al. 2010; See et al. 2010), diode array (Kueseng et al. 2009; Wang et al. 2010, 2011; Rodríguez-González et al. 2013) or mass spectrometry (Mazzella et al. 2009; van Pinxteren et al. 2009; Dujakovic et al. 2010; García-Galán et al. 2010; Postigo et al. 2010; Trtic-Petrovic et al. 2010; Lissalde et al. 2011) was also reported.

An extraction procedure to preconcentrate the analytes and remove possible interferences is mandatory to achieve the required levels. For this purpose, solid-phase extraction (SPE) is the preconcentration technique most commonly used for the determination of triazines in water samples (Kueseng et al. 2009; Mazzella et al. 2009; van Pinxteren et al. 2009; Bagheri et al. 2010; Dujakovic et al. 2010; García-Galán et al. 2010; Katsumata et al. 2010; Matamoros et al. 2010; Navarro et al. 2010; Postigo et al. 2010; Lissalde et al. 2011; Portoles et al. 2011; Rodríguez-González et al. 2013). However, this technique is being replaced by other fast techniques that minimise the waste of organic solvents according with the principles of green chemistry (Anastas and Eghbali 2010). Thus, some microextraction methods such as solid-phase microextraction (Mohammadi et al. 2009; Djozan et al. 2010), stir bar sorptive extraction (Sanchez-Ortega et al. 2009), liquid-phase microextraction (Trtic-Petrovic et al. 2010), liquid–liquid–solid microextraction (Hu et al. 2009b) and dispersive liquid–liquid microextraction (DLLME) (Zhou et al. 2009; Wang et al. 2010, 2011; Sanagi et al. 2012) have been applied for extraction and preconcentration of triazines in water as alternative to the SPE techniques. However, some of these new techniques have drawbacks like low sensitivity for the triazines studied (Mohammadi et al. 2009) and poor recoveries (Zhou et al. 2009; Wang et al. 2010), and in many cases, they are very laborious (Hu et al. 2009b; Trtic-Petrovic et al. 2010).

DLLME is based on the extraction of analytes in aqueous samples by employing an appropriated mixture of extraction solvent and disperser solvent to obtain a cloudy solution which assures a high contact surface and analyte extraction. The main problem is the adequate selection of the mixture of solvents because analytes losses can occur. This situation can be avoided by using an agitation step instead of adding the disperser solvent to achieve the formation of the cloudy solution (Zhang et al. 2011). The advantages of DLLME are simplicity of operation, short extraction time, low cost and high enrichment factors (Rezaee et al. 2006).

The aim of this work was the development of an environmental friendly, simple, fast and sensitive method for the simultaneous determination of seven triazine herbicides in estuarine seawater based on DLLME. The herbicides were determined by liquid chromatography with diode array detection (HPLC-DAD) and confirmed by liquid chromatography-electrospray ionisation tandem spectrometry analysis (LC-ESI-MS/MS). This method enables the determination of these pollutants at the levels required by European Union legislation (Council of the European Communities 2008a) using only 25 mL of sample; consequently, it can be an important tool to control the presence of triazines in seawater samples. The developed method was employed to determine the concentration of target compounds in seawater samples from estuary of A Coruña (Galicia, NW of Spain).

Materials and methods

Study area and sampling

The area studied is located in the coastline of Galicia (NW of Spain). Seawater samples were collected from ten potential polluted sites by triazines in estuary of A Coruña (see Fig. 1) during March of 2013. The estuary is formed by the mouth of Mero River, and it is also fed by many small rivers and brooks that traverse areas dedicated to agriculture. The sampling locations were selected by their proximity to one or more potential sources of contamination by triazine herbicides. The study area is illustrated in Fig. 1, and the ten sampling points and the source in brackets are listed below: 1 (tram line), 2 (golf course), 3 (a railway line and vegetable gardens), 4 (vegetable gardens), 5 (mouth of a river flowing through a golf course and growing areas), 6 (mouth of the Mero river that traverses areas dedicated to agriculture), 7 and 8 (residential areas with gardens and parks), 9 (mouth of two rivers flowing through a campsite, vegetable gardens and residential areas with gardens) and 10 (lake with access to the sea which receives two rivers that cross growing areas, vegetable gardens and residential areas with gardens). At each sampling point, three samples were collected.

Seawater samples were collected in amber glass containers, filtered through 0.6-μm glass fibre MN GF-6 filters from Macherey Nagel (Düren, Germany) to eliminate suspended solid matter. Due to the low stability of triazines, the samples were analysed the day of sampling.

Furthermore, unpolluted seawater samples from the Riazor beach at the city of A Coruña (Galicia, NW Spain) were used for the optimisation and validation of the method.

Standard and reagents

All herbicides analytical standards were supplied by Sigma-Aldrich (Inc. St. Louis, MO, USA). The individual stock standard solutions of 1,000 mg L^{-1} were prepared in methanol by exact weighing of high-purity substances and stored at −18 °C in the dark. Then, a mixture of all the compounds was prepared in methanol containing 10 mg L^{-1} each individual triazine and stored at −18 °C. All working solutions were daily prepared by appropriate dilution of the 10 mg L^{-1} standard solution.

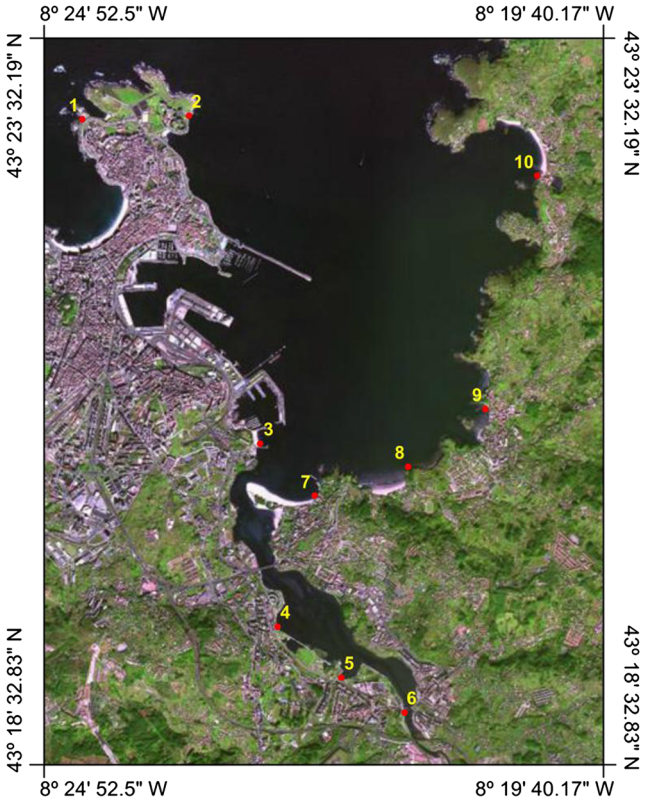

Fig. 1 Location of sampling sites in estuary of A Coruña (Galicia, NW of Spain)

1-Octanol Chromasolv® (grade HPLC 99 %) was purchased from Sigma-Aldrich Co. (Madrid, Spain); methanol was a superpurity solvent from Romil (Cambridge, UK), and acetonitrile was from Panreac (Barcelona, Spain). Ultrapure water was obtained using a Millipore Milli-Q system (Millipore, Bedford, MA, USA).

Apparatus

The HPLC-DAD system consisted of a 2695 pump with a 996 diode array detector from Waters (Milford, MA, USA). The LC-ESI-MS/MS analyses were performed using an Agilent HP-1200 Series LC system equipped with an autosampler, a binary solvent pump and a thermostated column oven. The LC system is coupled to a mass spectrometer with a triple quadrupole detector (API 3200, Applied Biosystem, Carlsbad, CA, USA) equipped with an APCI/ESI source.

The column was a stainless steel column (150×4.6 mm ID, particle size 5 μm) packed with Hypersil GOLD C_{18} chemical bonded phase from Thermo Scientific (Austin, TX, USA).

Extraction procedure

Aliquots of 25 mL of filtered seawater samples were extracted as follows—300 μL of 1-octanol was added as extractant solvent, and the mixture was vigorously shaken using an agitation plate Vibrax-VXR by IKA (Staufen, Germany) for 10 min at 1,200 rpm. The separation of both phases was performed by centrifugation (Eppendorf 5804, Madrid, Spain) at 3,500 rpm for 3 min. The droplets of 1-octanol were collected, and the volume was adjusted to 1 mL with methanol due to the immiscibility of the 1-octanol with the mobile phase of liquid chromatography. To remove any solid particles that might interfere in the analysis and damage the equipment, the extract was passed through a 0.2 μm syringe filter of PTFE (Teknokroma, Barcelona, Spain) before LC injection. The schematised DLLME procedure is shown in Fig. 2.

Chromatographic analysis

The HPLC-DAD analysis was carried out using the following acetonitrile/H_2O gradient elution: acetonitrile initial percentage of 30 % (8 min), increased linearly to 40 % in 5 min; increased to 50 % in 5 min, after which the percentage was returned to the initial conditions in 9 min. A constant mobile phase flow rate of 1 mL min^{-1} and 20 μL of sample volume were used.

The absorbance was measured continuously in the 200–400 nm range, and peaks areas quantification were carried out at 222.7 nm in order to achieve maximum sensitivity. All triazine herbicides were identified initially by retention time and then by applying spectral contrast techniques (incorporated in Millenium[32] software) the homogeneity of the spectral peak was confirmed. Finally, a spectral identification was carried out contrasting the spectrum with a standard library created in wavelength interval of 200–400 nm.

The LC-ESI-MS/MS analysis of the herbicides was performed in the positive ionisation mode. Data acquisition was performed in the multiple reaction monitoring mode, recording the transition between the precursor ion and the two most abundant product ions for each target analyte listed in Table 1.

Results and discussion

DLLME optimisation

The optimisation of the DLLME procedure was carried out by using seawater sample spiked with 10 μg L^{-1} of each triazine, and three replicates were used for all assays.

Several considerations must be taken into account to select an adequate extractant solvent in DLLME. The application is intended for a group of triazines in seawater matrices; therefore, the solvent must be immiscible with water. Several organic solvent (200 μL) were initially selected to develop the DLLME application: 1-octanol, dichloromethane and hexane. The organic solvent tends to form a single drop when it is added to a water sample; therefore, an agitation step (10 min,

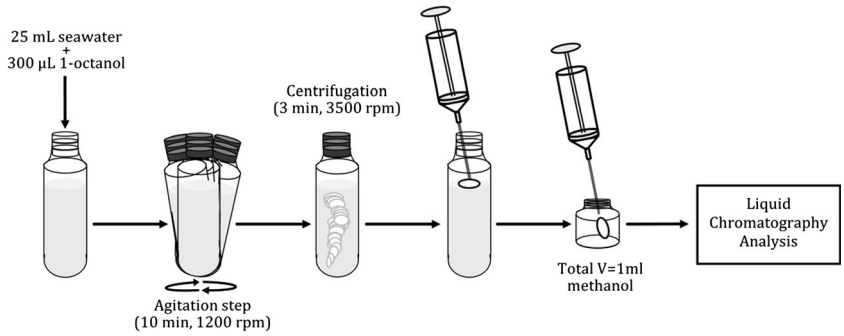

Fig. 2 Scheme of DLLME procedure

1,200 rpm) was needed to break down the drop of organic solvent and improve the dispersion process. The mixture was then centrifuged (3 min at 3,500 rpm) in order to separate the phases. After centrifugation, it was observed that the hexane was not able to form the drop, while, with the other solvents, a drop was formed at the bottom (dichloromethane) or top (1-octanol) of the test tube. Dichloromethane was discarded because the drop obtained is more difficult to collect and furthermore, according to principles of green chemistry, for being a chlorinated solvent. Consequently, 1-octanol was the selected solvent.

The effect of extraction time on the extraction efficiency in DLLME method was studied over the range of 3–60 min. For this purpose, 30 mL of a seawater sample solution containing 10 μg L^{-1} of each triazine were extracted using 200 μL of 1-octanol and 1,200 rpm. The results (see Fig. 3a) showed that the extraction efficiency slightly increased up to 10 min and kept constant until 40 min, and a small decrease was achieved when the extraction time is too long (60 min). Therefore, 10 min was selected as the optimum extraction time.

The effect of the extraction solvent volume was investigated using different volumes of 1-octanol (100, 200 and 300 μL) keeping constant the other experimental conditions. The results obtained (see Fig. 3b) showed that the extraction efficiency increased with solvent volume being the recoveries obtained with 300 μL of 1-octanol higher than 90 % for all compounds except for simazine (67 %), cyanazine (72 %) and symetrin (77 %). Consequently, 300 μL was chosen as optimum extraction solvent volume. With a view to improving the recovery of simazine, cyanazine and symetrin, a study using 25 mL of seawater sample solution containing 1 μg L^{-1} of each triazine was carried out. The recoveries obtained were satisfactory for all triazines (>80 %).

To evaluate the effect of stirring rate, 25 mL of a seawater sample solution containing 10 μg L^{-1} of each triazine were extracted for 10 min with 300 μL of 1-octanol. The stirring rate varied in the range of 800–1,400 rpm. As expected, a high stirring rate resulted in greater extraction efficiency obtaining the best results at 1,400 rpm (see Fig. 3c). However, because high stirring rate could break the tube and the signal variation for simazine and cyanazine between 1,200 and 1,400 rpm is very small, 1,200 rpm was selected as stirring rate for sample extraction.

Table 1 Optimized conditions for LC-ESI-MS/MS analysis of the investigated herbicides

Analyte	t_R (min)	Transitions (*m/z*) Precursor ion→product ion	Declustering potential (V)	Collision energy (V)
Simazine	6.62	202→104	41	33
		202→68	41	45
Cyanazine	7.23	241→214	46	21
		241→68	46	53
Simetryn	9.48	214→68	41	47
		214→124	41	29
Atrazine	9.94	216→174	41	23
		216→68	41	49
Ametryn	13.31	228→186	41	23
		228→68	41	51
Propazine	13.70	230→146	41	33
		230→58	41	37
Terbuthylazine	14.82	230→174	36	21
		230→68	36	49

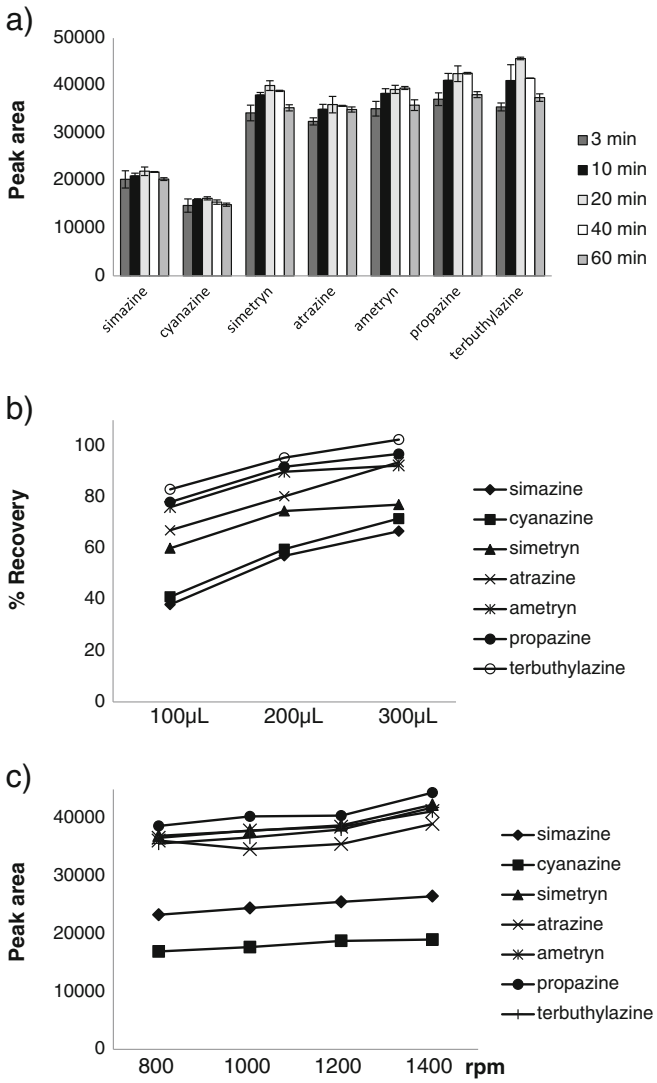

Fig. 3 **a** Effect of extraction time on the extraction efficiency in DLLME (n=3); **b** effect of extraction solvent volume in DLLME (n=3), and **c** effect of stirring rate on the signal (n=3)

The influence of the matrix on the peak area of the compounds was evaluated since sensitivity, reproducibility and accuracy can be greatly affected. The matrix effect was studied by comparison of the slopes of the calibration curves in methanol–octanol (7:3) and in the extract obtained after the DLLME procedure. The results showed that there are not slope deviation between matrix calibration and solvent calibration.

Furthermore, it is known that the addition of NaCl into the sample solution leads to increase ionic strength of sample solution and enhanced the extraction efficiency of triazine herbicides. Several authors have pointed out this salting-out effect on the extraction of triazine herbicides. So, Sanagi et al. (2012) developed a DLLME method for determination of simazine, atrazine and cyanazine in lake and tap water adding NaCl 5 % (*w/v*). Ye et al. (2007) employed 15 % (*w/v*) of NaCl to determine simazine, atrazine and cyanazine by single-drop microextraction in tap and reservoir waters, and Wang et al. (2011) used 10 % (*w/v*) of NaCl to analyse simazine, atrazine and ametryn in reservoir, river and well water. In this work, it was not necessary to study the influence of salt addition because the matrix studied was seawater.

On the other hand, since the triazines are ionisable compounds, the pH of sample solution can also affect the extraction efficiency. So, Ye et al. (2007) studied the effect of the pH on the extraction efficiency of triazines in water samples and reported that highest recoveries were achieved at the pH range of 5–9. Therefore, during experimental development, the pH of the samples was not adjusted because seawater shows a pH at the range 7.5–8.4.

Method validation

The method was validated in terms of linearity, recoveries, precision and limits of detection and quantification. All quantitative results were calculated using 25 mL of unpolluted seawater sample spiked with a standard mixture of the compounds. The results obtained are presented in Table 2.

Table 2 Analytical characteristics of the DLLME-HPLC-DAD method

Compound	LOD (μg L^{-1})	LOQ (μg L^{-1})	Determination coefficient (R^2)	[a]Intercept interval	[a]Slope interval	[a]CV Slope	Repeatability [b]RSD (%)	Reproducibility [b]RSD (%)	Recovery (%)
Simazine	0.37	1.10	0.9975	604±0.34	2086±0.09	0.0044	1.8	3.9	81±0.4
Cyanazine	0.13	0.40	0.9996	69±0.11	1397±0.03	0.0019	1.8	8.0	81±0.6
Simetryn	0.11	0.33	0.9997	-9±0.09	3242±0.02	0.0007	2.2	5.4	86±2.7
Atrazine	0.06	0.19	0.9999	-2±0.05	3422±0.01	0.0004	2.0	8.6	86±2.0
Ametryn	0.09	0.27	0.9998	-161±0.08	3082±0.02	0.0006	4.1	5.8	96±0.8
Propazine	0.15	0.47	0.9995	-17±0.13	3188±0.03	0.0010	4.5	6.4	94±2.0
Terbuthylazine	0.14	0.43	0.9996	-134±0.12	2968±0.03	0.0010	2.6	8.3	102±2.5

[a] Confidence level 95 %

[b] n=10 and n=5 for repeatability and reproducibility respectively (2 μg L^{-1})

The linearity of the matrix calibration curves was evaluated by triplicate analysis at six different concentration levels (1, 2, 3, 4, 5 and 6 μg L^{-1}). The calibration data obtained in this study are shown in Table 2. As it can be seen, very good linearity was obtained for all compounds with r^2>0.999, except for simazine which showed a determination coefficient only slightly lower (r^2=0.9975). The limits of detection (LODs) were determined as $3\times S_{y/x}/b$ and the limits of quantification (LOQs) as $10\times S_{y/x}/b$, where $S_{y/x}$ is the residual standard deviation and b is the slope of the matrix calibration curves. As it can be seen in Table 2, the detection and quantification limits obtained (between 0.06–0.37 and 0.19–1.12 μg L^{-1}, respectively) were adequate, being the LOQs much lower than 30 % of the parametric value requested by the legislation for surface water, except for simazine which showing about half of the legislated value (Council of the European Communities 2008a).

By comparing the LODs obtained by the proposed method with those reported in the literature using DLLME for the extraction of triazines in water, a better sensitivity is attained respect to Wang et al. (2010), who studied the limits of detection employing deionised water instead of water sample. Zhou et al. (2009) have obtained better and worse LODs for simazine and atrazine, respectively, using deionised water instead of a water sample. Wang et al. (2011) studied the extraction of triazines in river, reservoir and well water, and the LODs obtained are better for simazine and ametryn but worse for atrazine. It should be noted that the calculation of LODs was based on a signal-to-noise ratio (S/N) of 3. On the other hand, Nagaraju and Huang (2007) and Sanagi et al. (2012) have shown better LODs for the three triazine herbicides studied in each case (except for atrazine in the first case); however, it is important to take into account that the analysis was performed by gas chromatography coupled to mass spectrometry.

Repeatability, intermediate precision and accuracy were investigated by analysis of unpolluted seawater samples spiked at 2 μg L^{-1}. The spiked concentration was selected to test the method performance at the residue level set by European Union Legislation for atrazine in surface waters (Council of the European Communities 2008a). The repeatability was calculated as within-day RSD of peak areas using ten replicates analysed in the same day and by the same analyst. In the case of intermediate precision, five replicates were analysed in consecutive days and by the same analyst, and it was calculated as between-day RSD of peak areas. As it can be seen in Table 2, the results obtained were satisfactory with RSD values below 5 % and 10 % for repeatability and intermediate precision, respectively.

Accuracy of the method was evaluated in terms of recovery. The results obtained for five replicates of the sample spiked with the triazine herbicides are presented in Table 2. The results demonstrated that the method achieved satisfactory recoveries in the range of 81–102 %, with associate standard deviations below 3 % for all compounds. Furthermore, the obtained recoveries and relative standard deviation are comparable (Wang et al. 2011; Sanagi et al. 2012) or even much better (Zhou et al. 2009; Wang et al. 2010; Nagaraju and Huang 2007) to those provided by other authors for the determination of some of these pollutants by DLLME in waters.

The advantages of this method when compared with other ones described in the bibliography are: (1) The use of agitation step for dispersion instead of a disperser solvent avoids the main DLLME problem that is the adequate selection of the mixture of solvent/dispersant because analytes losses can occur; (2) in this study, seven triazines are determined whereas in other studies using DLLME only between two and five triazines are analyzed, except in Nagaraju et al. which determined eight triazines but only four of them matched up with triazines of the proposed method (simazine, atrazine, propazine and simetryn) and (3) the method is validated for seawater while other authors applied their methods to other types of surface waters (farm, lake, groundwater, river and

reservoir waters) or tap water (Nagaraju and Huang 2007; Zhou et al. 2009; Wang et al. 2010, 2011; Sanagi et al. 2012).

Therefore, it can be concluded that the proposed method could be a competitive analytical tool for the analysis of triazines at trace levels in seawater samples in compliance with EU directives. As an example, Fig. 4 shows the chromatograms corresponding to unspiked and spiked seawater sample (2 μg L^{-1}) extracted using the DLLME method studied.

Application of the method to the analysis of estuarine seawater samples

The proposed method was applied to analyse the target compounds in ten water samples from the estuary of A Coruña (Galicia, NW of Spain). The sampling took place during March of 2013. The analysis showed that the concentration of the triazines were below the LODs.

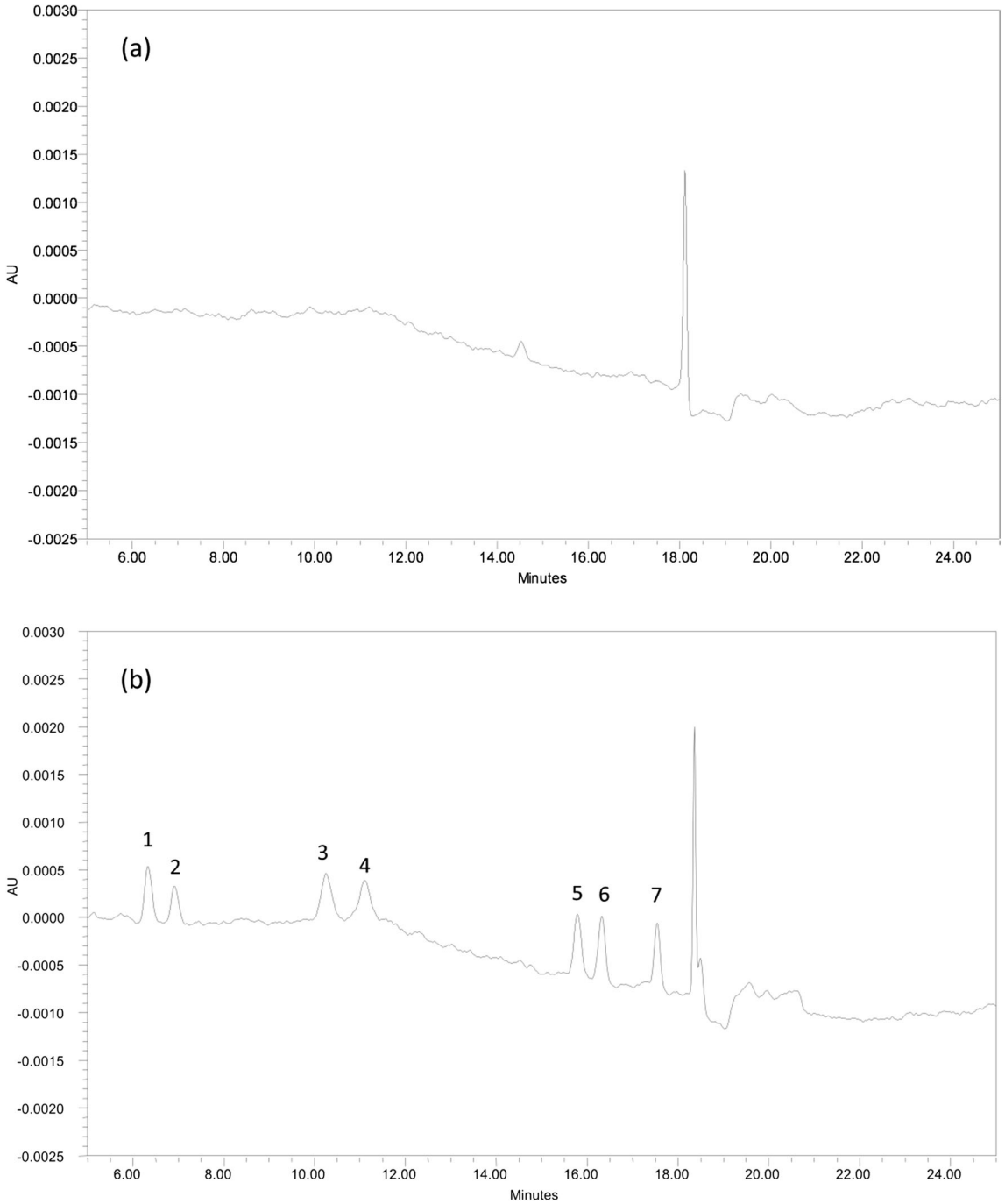

Fig. 4 Chromatograms obtained after DLLME **a** seawater sample, **b** seawater sample spiked at 2 μg L^{-1}. Target compounds are numbered as follows: (*1*) simazine, (*2*) cyanazine, (*3*) simetryn, (*4*) atrazine, (*5*) ametryn, (*6*) propazine, (*7*) terbuthylazine

Conclusions

A fast and simple method for the analysis of seven triazines from seawater samples has been developed. The method is based on DLLME prior to HPLC-DAD identification and quantification. Confirmation with LC-ESI-MS/MS was taken into account.

The method uses small volumes of organic solvent in agreement with the principles of the green chemistry and agitation instead of dispersive solvent which simplifies the experimental procedure.

The developed method has shown suitable precision and accuracy for determination of triazines in seawater samples. The limits of quantification enable the determination of these pollutants at the levels required by European Union legislation (Council of the European Communities 2008a) using only 25 mL of sample; consequently, it can be an important tool to control the presence of triazines in estuarine seawater samples. Furthermore, it is important to note that to the best of our knowledge no previous studies have been published to the determination of these seven triazines in seawater.

The proposed method was applied to determine the target compounds in seawater from estuary of A Coruña (Galicia, NW of Spain). Although the triazines under study have not been detected in the samples analysed, the monitoring of their levels in marine ecosystems is of great economic and environmental importance. It is important to take into account that measurements of known quality represent the foundation of the water quality evaluation system and the basis for decisions to be taken to achieve Marine Strategy Framework Directive and environmental objectives at the end of 2015 (Council of the European Communities 2008b).

Acknowledgments The authors acknowledge the financial support of the "Ministerio de Educación y Ciencia" (Project CTQ2007-63949/BQU) and the "Xunta de Galicia" (Project 09MDS038103PR and "Ayuda Consolidación e Estructuración de Unidades de Investigación Competitivas"). N. Rodríguez-González also thanks the "Xunta de Galicia" for the concession of her award under the program "Campus del Mar."

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ANEXO I-III

On-line solid-phase extraction method for determination of triazine herbicides and degradation products in seawater by Ultra-Pressure Liquid Chromatography-Tandem Mass Spectrometry. *Journal of Chromatography A* (2016) 1470, 33-41.

Journal of Chromatography A, 1470 (2016) 33–41

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

On-line solid-phase extraction method for determination of triazine herbicides and degradation products in seawater by ultra-pressure liquid chromatography–tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 18 June 2016
Received in revised form
30 September 2016
Accepted 5 October 2016
Available online 6 October 2016

Keywords:
Triazines
Degradation products
Seawater
Ultra-pressure liquid chromatography
Mass spectrometry

ABSTRACT

A fast, simple, selective and sensitive method has been developed for the determination of nine triazine herbicides (ametryn, atrazine, cyanazine, prometryn, propazine, simazine, simetryn, terbuthylazine and terbutryn) and eight degradation products (desethyl atrazine, desethyl-desisopropyl atrazine, desethyl 2-hydroxyatrazine, desethyl terbuthylazine, desisopropyl atrazine, desisopropyl 2-hydroxyatrazine, 2-hydroxyatrazine and 2-hidroxyterbuthylazine) in seawater samples. On-line solid-phase extraction coupled with ultra-pressure liquid chromatography-tandem mass spectrometry was employed for simultaneous analysis of all compounds in 11 min. Validation parameters were studied through the estimation of the limits of detection and quantification, calibration curves and precision. Limits of quantification ranged from 0.023 to 0.657 $\mu g\,L^{-1}$. Good linearity was obtained for all compounds with $R^2 > 0.99$ in all cases. Furthermore, inter-day precision (0–2.1%) and intra-day precision (0–3.9%) were shown to be satisfactory. On-line solid-phase extraction recoveries in spiked unpolluted seawater sample were evaluated and acceptable values (80.3–99.8%) with adequate RSD (0.1–3.1%) were found.

Finally, the proposed method was applied to the analysis of the target compounds in seawater samples collected from seawater nearby a zone of intensive horticulture of Matosinhos (Portugal). The concentrations of the herbicides were below the limit of detection in all cases.

1. Introduction

The occurrence of micropollutants has been highlighted in thousands of publications during the last decade, which have pointed out a growing concern about them. Although there are no discharge limits for most micropollutants, some regulations have been published. Many of these substances, including triazine herbicides, are toxic and hazardous; as an example, atrazine produces genotoxic damage in fish species [1]. Therefore, the chemical pollution of surface water can affect the environment, several effects as chronic toxicity on aquatics organisms, accumulation in the ecosystem, as well as injures in human health are described. The European Union has included simazine and atrazine in the list of 33 priority substances in the EU Water Framework Directive (2000/60/EC) by way of Decision 2455/2001/EC [2]. Moreover, the Directive 2008/105/EC [3] sets the Environmental Quality Standards (EQS) for these compounds in surface water. Recently, the Directive 2013/39/EU amending the Directives 2000/60/EC and 2008/105/EC includes terbutryn to the list of priority substances [4]. This Directive establishes a maximum permitted concentration of 0.34, 2 and 4 $\mu g\,L^{-1}$ for terbutryn, atrazine and simazine respectively. Furthermore, Directive 2013/39/EU calls the attention on the important role of monitoring emerging pollutants that are not regularly considered in monitoring programs but can have toxicological effects.

In this way, studies examining the concentration of triazines in surface waters have expanded the list of compounds including their main degradation products [5–7]. These degradation products are produced through abiotic and biotic processes in the soil, groundwater and surface water [8]. Because of their mobility in the soil-water environment, the degradation products can reach water bodies more easily than triazines; thus, the impact due to herbicides tends to be underestimated when only the triazines are analysed

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http://dx.doi.org/10.1016/j.chroma.2016.10.007
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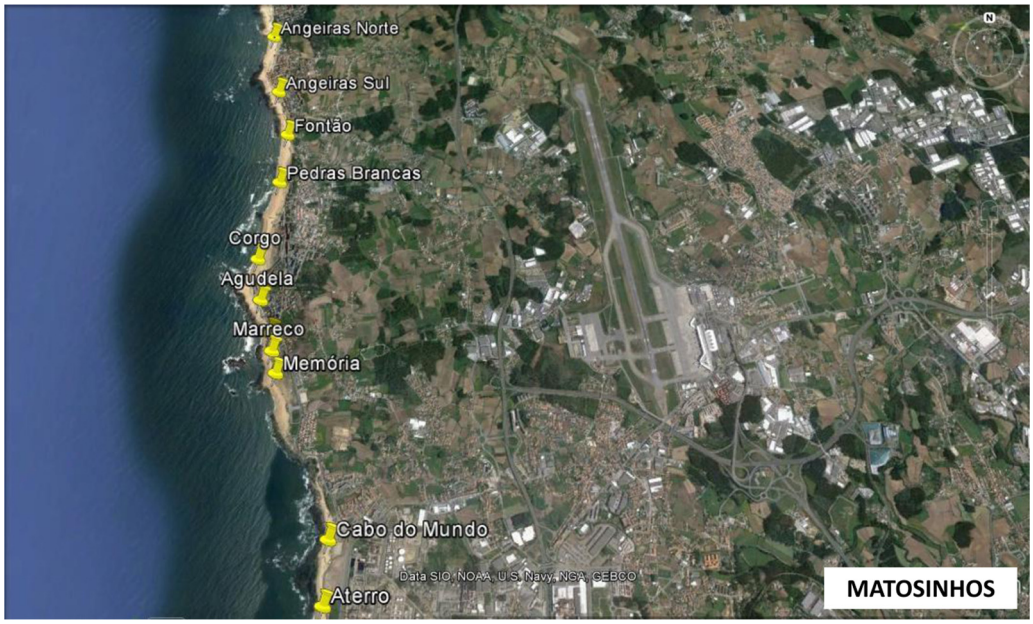

Fig. 1. Location of sampling sites in Matosinhos (North of Portugal).

in samples. Therefore, the main degradation products should be included in current analytical methods to obtain a better knowledge of water quality regarding herbicides contamination [9].

In order to support the implementation of the Directive 2013/39/EU, simple, selective and sensitive analytical methods are required. Different chromatographic techniques have been used to determine triazines and/or their degradation products in water. Between these techniques, the application of liquid chromatography-tandem mass spectrometry (LC–MS/MS) has provided an increased selectivity and sensitivity [10–16]. The recent development of chromatographic columns using new stationary phases with particle size <2 μm for ultra-pressure liquid chromatography (UPLC) allows significantly shorten the analysis time [17]. UPLC–MS/MS can offer not only good sensitivity but also high confidence in the confirmation of compounds detected allowing to achieve more than 3–4 identification points. Thus, UPLC coupled to tandem MS provides an interesting tool for fast determination of these compounds in water samples [18–21].

Regarding extraction procedure, off-line SPE methods usually require high sample volume and are time-consuming [22]. On the other hand, although on-line SPE provides advantages, it also has disadvantages including the complexity of the set-ups of valve-switching, lack of flexibility as compared to off-line SPE and possible interferences from loading the entire extracted sample. Most of these problems have been resolved with advances in automated on-line SPE systems, integrated and flexible software programs and application of tandem spectrometry detectors for better selectivity. On-line SPE offers advantages, such as the minimal amount of solvents required for extraction, fast sample preparation and small sample volumes [23]. For this reason, the use of on-line SPE coupled to liquid chromatography-mass spectrometry has increased and some methods have been published for the analysis of triazines and their degradation products in water [7,13,20]. Nonpolar SPE sorbents are generally selected for extracting triazines from water samples. However, the degradation products, which contain polar functional groups, can be more efficiently extracted by using polar sorbents [24]. For SPE, different solid phases such as Oasis MCX cartridges [25,26], PLRP-s [13,20], Oasis HLB cartridges [15,20], Amberlite XAD-4 resin [27] and Oasis MAX cartridges [16] have been employed for triazines and their major hydroxy and dealkylated products. Several articles have compared different adsorbents and Oasis HLB has shown to have better ability to retain both non-polar and polar compounds.

The aim of this study was to develop a fast, simple, selective and sensitive analytical method for the quantification and confirmation of 17 compounds (nine triazine herbicides as well as their main degradation products) in seawater. The method developed is based on an on-line solid-phase extraction (OASIS HLB cartridges) followed by UPLC–MS/MS determination. This method enables the determination of these pollutants at the levels required by European Union legislation [4]; consequently, it can be an important tool to control the presence of triazines and their degradation products in seawater samples. To the best of our knowledge, studies using on-line SPE-UPLC–MS/MS have not been done to determine the target compounds in seawater. However, the monitoring of these compounds in marine ecosystems situated close to areas of intensive horticulture is of great interest. Finally, the proposed method was applied to determine the target herbicides in seawater samples from beaches of Matosinhos (Portugal).

2. Experimental

2.1. Study area and sampling

The studied area is situated in the Portuguese seashore. This area presents some vulnerability due to hydrogeological factors and the interdependence with properties of pesticides what may add higher importance to the groundwater contamination and in this particular case reaching seawater [28]. Fig. 1 shows the study area with the sampling points. Seawater samples were collected from ten beaches susceptible to contamination by triazines during December of 2015. Sampling points are listed below: 1 (Angeiras Norte), 2 (Angeiras Sul), 3 (Fontão), 4 (Pedras Brancas), 5 (Pedras do Corgo), 6 (Agudela), 7 (Marreco), 8 (Memória) 9 (Cabo do Mundo) and 10 (Aterro).

Seawater samples were collected in amber glass bottles and transported to the laboratory under cooled conditions (4 °C). At each sampling point, three samples were collected. Upon reception, samples were filtered through a PTFE syringe filter to eliminate

suspended solid matter. Due to the low stability of triazines, the samples were analysed the day of sampling.

Furthermore, unpolluted seawater samples from the Circunvalação beach at the city of Porto were used for method validation.

2.2. Chemicals and materials

All herbicides analytical standards (purity between 97.5 and 99.5) were supplied by Sigma-Aldrich (Inc. St. Louis, MO, USA). The structures of the target compounds are shown in Fig. 2. The individual stock standard solutions of 1000 mg L^{-1} were prepared in methanol by exact weighing of high-purity substances and stored at −18 °C.

Mixed stock solution of 17 compounds was prepared in methanol containing 1 mg L^{-1} for desethyl-desisopropyl atrazine (DEDIA), desethyl 2-hydroxyatrazine (DEHA), desisopropyl atrazine (DIA), desisopropyl 2-hydroxyatrazine (DIHA) and 2-hydroxyterbuthylazine (HT); and 0.1 mg L^{-1} for the nine triazines, desethyl atrazine (DEA), desethyl terbuthylazine (DET) and 2-hydroxyatrazine (HA). All working solutions were daily prepared by appropriate dilution of the mixed solution with methanol.

Methanol for liquid chromatography was supplied by J.T. Baker (Deventer, the Netherlands), and Milli-Q water was obtained from a purification system from Millipore (Molsheim, France). Ammonium acetate (≥99%) (LC grade) was purchased from Sigma-Aldrich (Inc. St Louis, MO, USA).

OASIS® HLB Direct connect HP columns 20 μm (2.1 × 30 mm) were supplied by Waters (Milford, MA, USA). PTFE syringe filters (0.2 μm pore size) were purchased from Merck (Darmstadt, Germany).

2.3. Ultra-pressure liquid chromatography

The pesticides and metabolites were separated by an Acquity system (Waters, Manchester, UK) consisting of an Acquity UPLC™ binary solvent manager, an Acquity UPLC™ sample manager and an Acquity UPLC™ heater equipped with an Acquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm particle size) with a pre-column (UPLC BEH C18 2.1 mm × 5 mm, 1.7 μm particle size). These columns are packed with a C18 reverse-phase bounded to ethylene-bridged hybrid (BEH) substrate. Elution was performed under gradient mode using 5 mM ammonium acetate in water as mobile phase A and 5 mM ammonium acetate in methanol as mobile phase B. Separation was carried out at 40 °C using a flow rate of 300 μL min^{-1}. The gradient elution was performed as follows: mobile phase A initial percentage of 95% decreasing linearly to 0% in 5 min and holding in it for 3 min, after which the percentage was returned to the initial conditions in 0.1 min. Initial conditions were maintained during 3 min for the column re-equilibration. The sample volume injected into the UPLC system was 5 mL. The optimized method allowed the concurrent detection of 17 pesticides in a chromatographic run of 11 min.

2.4. Tandem mass spectrometry

An Acquity triple-quadrupole mass spectrometer (Acquity TQD™ Waters, Manchester, UK) fitted with an electrospray ionization source (ESI) was used for pesticide detection. MS/MS detection was performed in negative or positive mode, depending on the compounds. ESI mode was set individually for each target compound, the switch time between positive and negative mode was 20 ms. Data acquisition was performed under multiple reaction monitoring (MRM) mode, recording the transition between the precursor ion and the most abundant product ions for each target analyte. The optimization of MS/MS conditions, including the search for precursor and product ions, optimization of the sample cone voltage, collision energy was performed using an Intellistart® module of the MassLynx 4.1 software. MRM conditions used for the analysis of pesticides are shown in Table 1.

The ESI interface conditions were optimized for maximum intensity of the precursor ions as follows: capillary voltage 3.5 kV, source temperature 140 °C and desolvation temperature 350 °C. The nebulizer and desolvation gas flows were set at 850 and 50 L h^{-1} respectively. Nitrogen was used as nebulizer and desolvation gas. Argon was used as collision gas at a pressure of 3.7×10^{-3} mBar. The analytical device was controlled by MassLynx software (version 4.1 SCN 714) and the data were processed using QuanOptimize software.

2.5. On-line solid phase extraction

The SPE was performed by a CTC hTC PAL autosampler (fitted with a 5 mL syringe, a 5 mL loop and a sample rack of 32 × 10 mL) coupled to the UPLC–MS/MS instrument. On-line SPE was carried out using a device, which employs a dual LC column system; one OASIS HLB column for pre-concentration and another for analytical separation (see Fig. 3). The extraction of all seawater samples, aqueous standard solutions and blanks was performed by loading 5 mL of the solution through an OASIS HLB on-line SPE cartridge previously conditioned with water/methanol (95:5, v/v) during 10 min (flow rate of 1 mL min^{-1}). The trap cartridge was fitted into the load position A of a valve switching Rheodyne 10 port. A Waters isocratic pump was used to load the sample at 5 mL min^{-1} onto the trapping cartridge. After sample loading (11 min), the valve was switched to position B being the analytes eluted from the cartridge to the LC column with the chromatographic mobile phase using a Waters Binary pump.

3. Results and discussion

3.1. UPLC–MS/MS development

The optimum cone voltage and collision energy for each pesticide was selected with the aim of obtaining the precursor ion and the MRM transition with the highest sensitivity and the other product ions (see Table 1 and Fig. 4). The most sensitive transition was chosen for quantification and the other transitions were used for confirmation. Optimization of MS/MS settings was performed by direct infusion of individual standard solutions (0.1–0.5 μg mL^{-1} in methanol). Negative or positive mode was studied for all analytes. The analysis of cyanazine was performed in the negative mode and the determination of the remaining compounds was performed in the positive mode. The manufacturer recommended values for desolvation and cone gas flow were selected. The suitable setting of the instrument for each pesticide is shown in Table 1.

Once MS parameters were established, the UPLC conditions were optimized in order to attain an adequate elution of the target compounds and a short analysis time. The mobile phase composition was evaluated considering that chromatographic behaviour of some compounds was very similar and their separation was difficult. Furthermore, degradation products are strongly dependent on pH and the use of a mobile phase with a buffer or a modifier is necessary. For this reason, different mobile phases (water and methanol with and without ammonium acetate) were tested and the best chromatographic separation was achieved by using 5 mM ammonium acetate in both mobile phases. In view of the satisfactory results obtained, other modifiers as formic acid or acetic acid were not tested.

Various analytical gradients were also evaluated. Using the conditions described in section 2.3, all the compounds were ade-

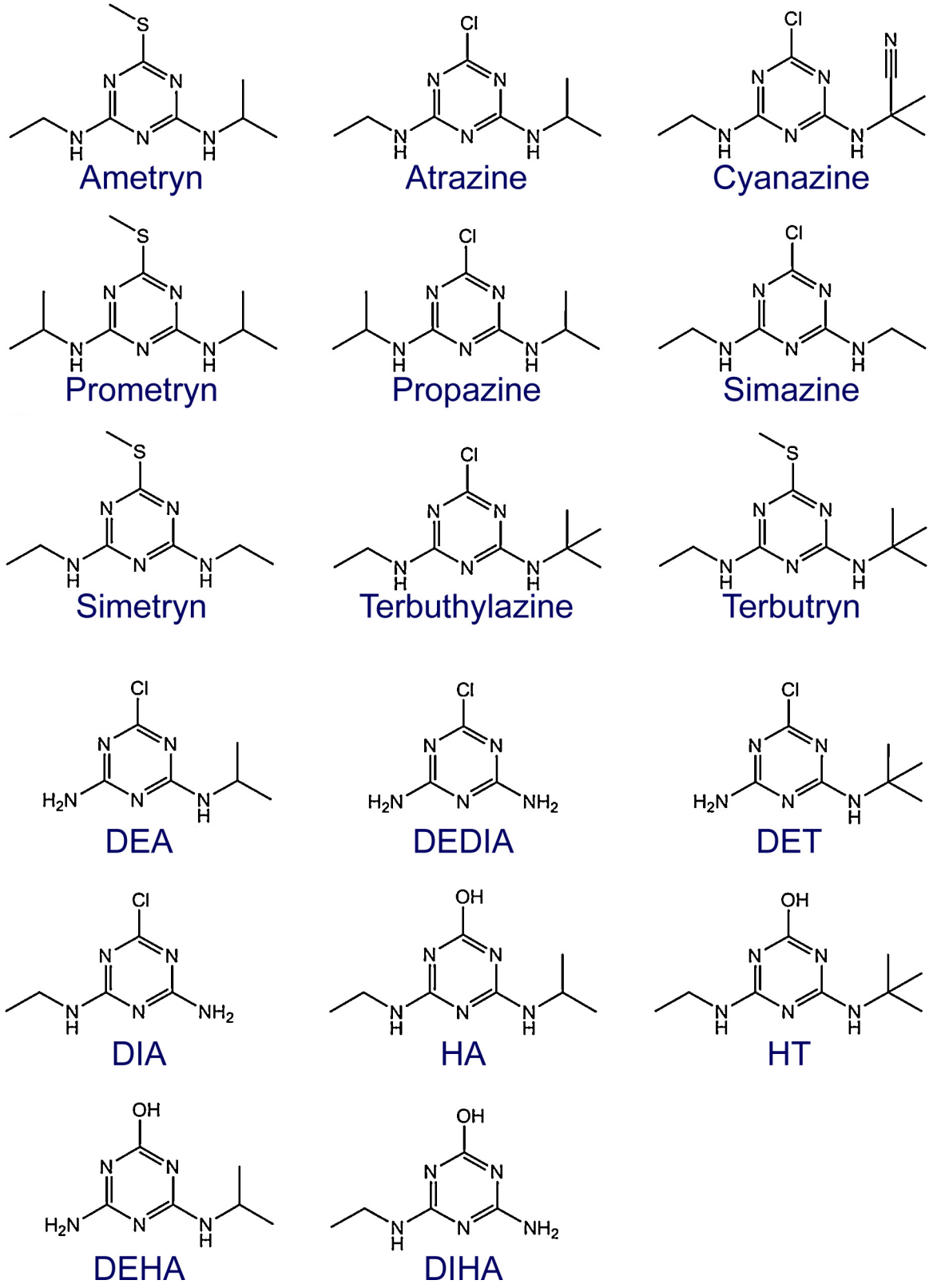

Fig. 2. Structures of the target compounds.

quately separated within a run time of 11 min. The chromatograms obtained from a spiked seawater sample with 0.1 $\mu g\,L^{-1}$ (the nine triazines, DEA, DET and HA) and 1 $\mu g\,L^{-1}$ (DEDIA, DEHA, DIA, DIHA and HT) are shown in Fig. 5.

3.2. Method performance

The analytical method is based on a method previously developed by the authors for the analysis of the nine target triazines in seawater using Oasis HLB by means of off-line SPE [29]. Fur-

Table 1
Retention time and MS/MS optimized parameters for the studied pesticides.

Compound	Rt (min)	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)	Cone (V)	Collision energy (eV)
DEHA	3.68	170,1	43,1	36	34
			86,0		22
			128,0		16
DIA	5.50	174,0	68,0	36	26
			96,0		16
			104,0		26
HA	5.55	198,1	69,0	42	36
			86,0		22
			114,0		22
DEA	5.85	188,0	79,0	50	16
			146,1		12
HT	5.92	332,1	234,1	12	14
			256,1		6
DIHA	5.92	156,1	69,0	32	24
			86,0		18
			114,0		14
Cyanazine	6.12	239,1	81,0	20	16
			176,1		14
			212,0		10
Simazine	6.19	202,5	104,0	38	28
			146,0		18
DET	6.22	202,1	79,0	34	22
			104,0		28
			146,0		14
DEDIA	6.24	146,1	43,0	38	24
			79,0		18
Atrazine	6.48	216,1	96,1	34	24
			174,1		20
Simetryn	6.58	214,1	68,0	42	32
			96,1		26
			124,1		20
Propazine	6.63	230,1	79,0	40	30
			104,0		34
			146,0		24
Terbuthylazine	6.64	230,1	146,0	36	22
			174,0		16
Ametryn	6.76	228,1	68,1	42	38
			91,0		26
			96,1		26
Prometryn	6.87	242,1	43,1	44	36
			68,1		34
			158,0		24
Terbutryn	6.95	242,1	91,0	34	26
			96,1		30
			186,1		18

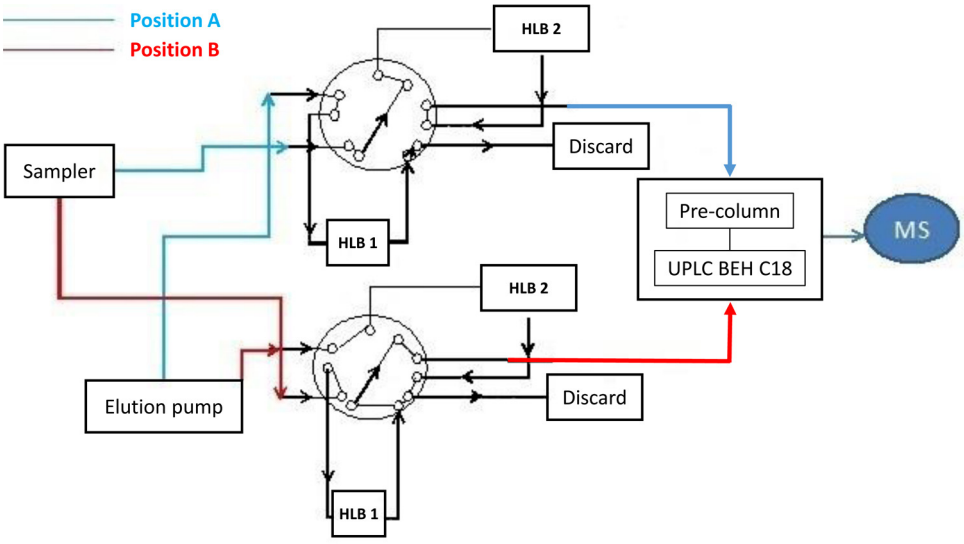

Fig. 3. Scheme of the on-line SPE-UPLC-MS/MS.

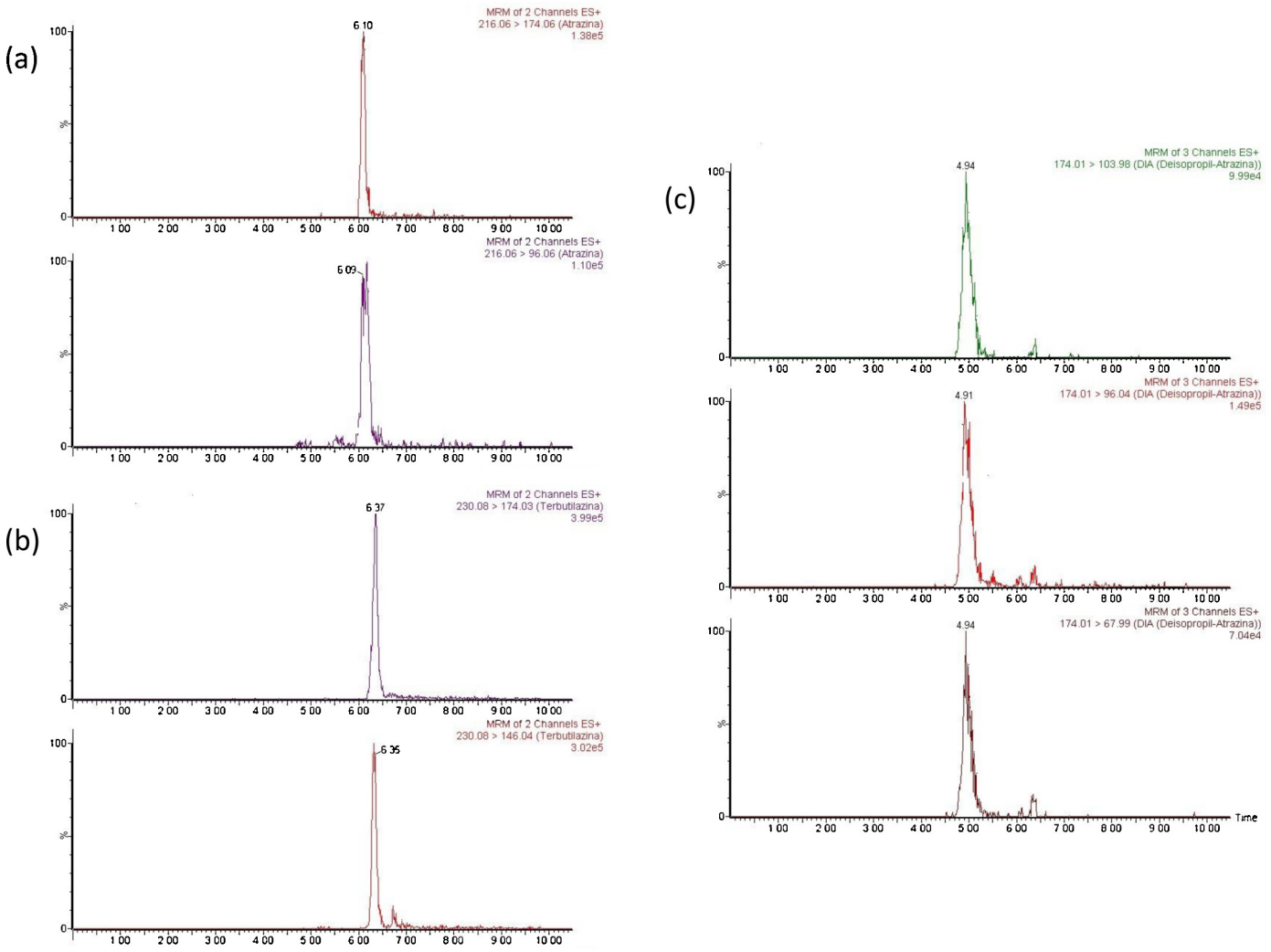

Fig. 4. MRM transitions (a) atrazine, (b) terbuthylazine and (c) DIA.

thermore, it was taken into account that Oasis HLB has shown to have better ability to retain some degradation products than other sorbents. Thus, Gervais et al. [18] studied different sorbent for multiresidue determination of pesticides in water including atrazine, cyanazine, DEA, DIA, simazine and terbuthylazine and the best recoveries were obtained by the extraction with Oasis HLB. Furthermore, Benvenuto et al. [19] have tested two sorbents Oasis HLB and Oasis MCX for the determination of atrazine, simazine, terbuthylazine, terbumeton, terbutryn and some of their main degradation products (DEA, DIA, DET, HA, HT) and Oasis HLB showed better ability to retain both non-polar and polar compounds.

Since the initial conditions assayed (described in Section 2.5) have shown to be adequate, more studies about parameters such as sample volume, sample loading flow and elution were considered unnecessary.

The method was validated by estimation of the linearity, trueness and precision, limits of detection and limits of quantification. The results obtained are presented in Table 2.

The linearity was evaluated using standard solutions analysed with the method proposed. Calibration curves were constructed using seven calibration points, with three replicates for each calibration level, at the concentration range shown in Table 2. As it can be seen, very good linearity was obtained for all compounds with coefficients of determination (R^2) higher than 0.999, except for DIHA and HA which showed a coefficient of determination only slightly lower (0.9987 and 0.9989 for DIHA and HA respectively).

The limits of detection (LODs) were calculated as $3*S_{y/x}/b$ and the limits of quantification (LOQs) as $10*S_{y/x}/b$, where $S_{y/x}$ is the residual standard deviation and b is the slope of the matrix calibration curves. As it can be seen in Table 2, the limits of detection and quantification obtained (between 0.008–0.020 and 0.023–0.061 $\mu g\,L^{-1}$ respectively) for triazine herbicides and three degradation products (DEA, DET and HA) were satisfactory. LOQs are much lower than 30% of the more restrictive parametric value requested by the legislation for triazines in surface water (maximum permitted concentration of 0.34 for terbutryn). For the remaining degradation products, the limits of detection and quantification (between 0.133–0.217 and 0.402–0.657 $\mu g\,L^{-1}$ respectively) were adequate, being the LOQs $\leq$ 30% of the parametric value requested by the legislation for atrazine in surface water [4]. Moreover, the selectivity of the method was evaluated by analysing control blank samples. The absence of any signal at the same retention time of the selected compounds indicated there were no any matrix interferences or contamination that can give a false positive signal.

The precision of the overall analytical procedure, expressed as relative standard deviation (RSD), was evaluated as intra-day and inter-day precision. To study intra-day precision, RSDs were calculated at two levels of concentrations measuring ten replicates at

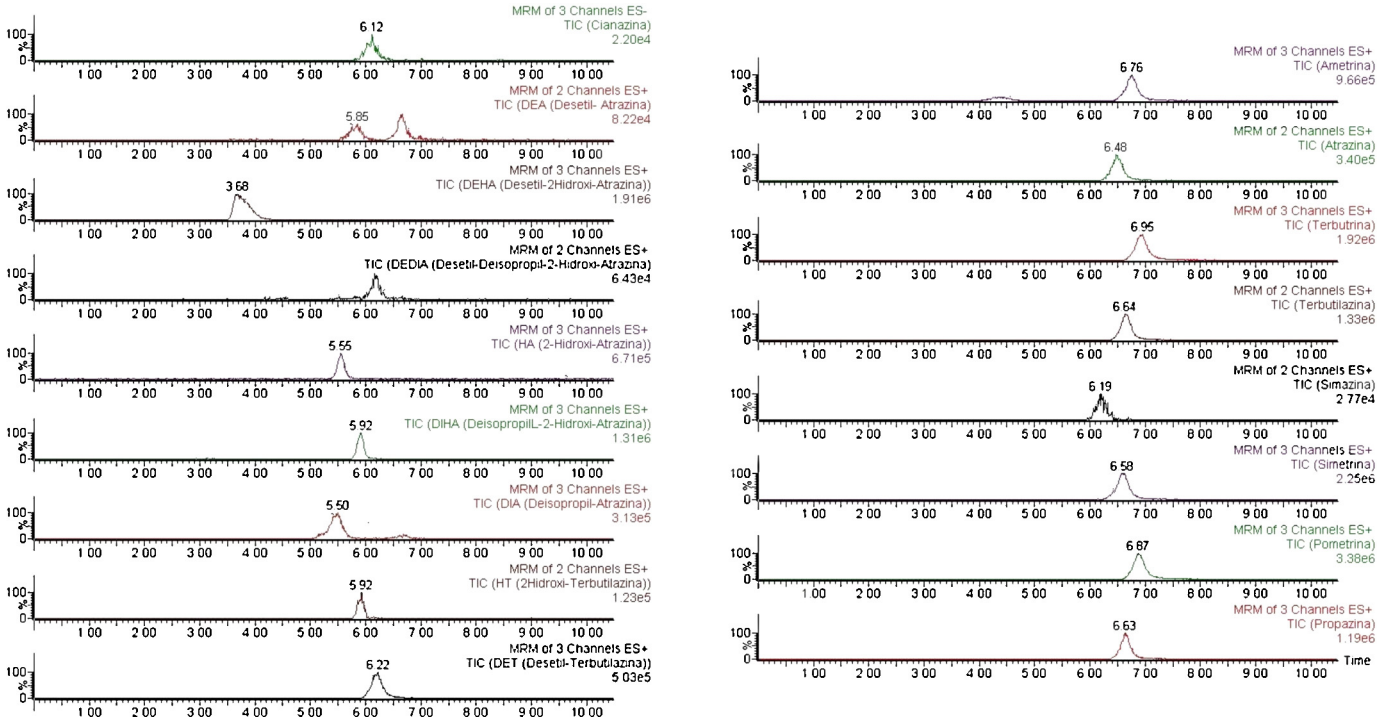

Fig. 5. UPLC–MS/MS chromatograms of herbicides and degradation products from a seawater sample spiked with 0.1 $\mu g\,L^{-1}$ for the nine triazines, DEA, DET and HA and 1 $\mu g\,L^{-1}$ for DEDIA, DEHA, DIA, DIHA and HT.

each concentration level during the same day. As it can be seen in Table 2, the obtained values were satisfactory for all compounds (RSDs lower than 4%). The inter-day precision was studied at one level of concentration by measuring three replicates on five consecutive days. The obtained values lower than or equal to 2.1% (see Table 2) indicate that the developed method was reproducible.

Trueness of the method was determined by spiking 5 mL of seawater sample at two concentration levels depending on the compound (0.1 $\mu g\,L^{-1}$ for the nine triazines, DEA, DET and HA and 1 $\mu g\,L^{-1}$ for DEDIA, DEHA, DIA, DIHA and HT). To evaluate the trueness, the analytical recoveries of spiked samples, using five replicates at each fortification level, were calculated. As it can be seen in Table 2, satisfactory recoveries for all compounds in the range of 80.3–99.8% were achieved with associate standard deviations below 3.1.

3.3. Application of the method to the analysis of seawater samples

The proposed method was applied to analyse the target compounds in seawater samples of ten beaches susceptible to contamination by triazines in the seashore of Matosinhos (North of Portugal). Although none of the seawater samples contained detectable amount of target triazines and degradation products, this study is part of a project in which a detailed study with sampling in spring, summer and early autumn could take into account. During these months, it is time of sowing common crops and mainly fumigating different crops and vineyards.

4. Conclusions

On-line SPE-UPLC–MS/MS method has shown to be a fast, sensitive and robust alternative method to traditional off-line SPE for the analysis of nine triazines and their main degradation products in seawater samples. The proposed method is suitable to be used in routine analysis due to sample pre-treatment is not required and allows rapid trace enrichment from low sample volume with minimal sample handling. Furthermore, on-line SPE reduces chemical waste due to the use of a minimal amount of extraction solvents. The method has shown suitable precision and good recovery values were obtained for all compounds. The limits of quantification enable the determination of these pollutants at the levels required by European Union legislation [4] using only 5 mL of sample. Consequently, it can be an important tool to control the presence of triazines and their degradation products at trace levels in seawater samples in compliance with EU directives. It is noteworthy that a few methods based on solid-phase extraction combined with UPLC–MS/MS have been used to measure triazines and degradation products in river waters; however, there are not studies in seawater. Furthermore, an important difference of the proposed method with previously described methodology for the analysis of triazines herbicides and their main degradation products is the determination of a greater number of degradation products simultaneously with triazines.

The method was applied to the analysis of the target compounds in seawater from the coastline of Matosinhos (North of Portugal). Although the triazines and degradation products under study have not been detected in the samples analysed, the monitoring of their levels in marine ecosystems situated close to areas of intensive horticulture is of great local interest both economic and environmental.

Funding

This work was supported by Ministerio de Economía y Competitividad (CTM2013-48194-C3-2-R) and Program of Consolidation and Structuring of Units of Competitive Investigation of the University System of Galicia (Xunta de Galicia) potentially cofounded by European Regional Development Fund (ERDF) in the frame of the operative Program of Galicia 2007–2013 (reference: GRC2013-047).

Table 2
Validation of the on-line SPE-UPLC–MS/MS method.

Compound	Concentration range ($\mu g L^{-1}$)	LOD ($\mu g L^{-1}$)	LOQ ($\mu g L^{-1}$)	Determination coefficient (R^2)	[a]Intercept interval	[a]Slope interval	[a]CV slope (%)	Intraday precision RSD (%)[b]		Inter-day precision RSD (%)[c]	Recovery (%)
								Level 1	Level 2		
Ametryn	0.025–0.5	0.017	0.051	0.9992	14051.3 ± 0.0	853516 ± 0	$3.7\ 10^{-6}$	0.6	0.1	0.1	86.8 ± 0.6
Atrazine	0.025–0.5	0.019	0.057	0.9990	6409.8 ± 0.0	351161 ± 0	$1.0\ 10^{-5}$	1.8	0.4	0.4	81.0 ± 1.8
Cyanazine	0.025–0.5	0.018	0.054	0.9991	125.9 ± 0.0	32941 ± 0	$1.0\ 10^{-4}$	1.9	0.5	0.8	90.0 ± 1.7
DEA	0.025–0.5	0.016	0.048	0.9993	1615.0 ± 0.0	118441 ± 0	$2.5\ 10^{-5}$	2.2	0.6	2.1	88.9 ± 0.6
DEDIA	0.25–5	0.152	0.459	0.9995	138.5 ± 0.1	6398 ± 0	$5.0\ 10^{-4}$	0.8	0.5	0.2	85.7 ± 2.3
DEHA	0.25–5	0.133	0.402	0.9995	3471.7 ± 0.1	63402 ± 0	$4.0\ 10^{-5}$	2.5	0.6	1.2	89.7 ± 0.1
DET	0.025–0.5	0.018	0.056	0.9991	16205.8 ± 0.0	582235 ± 0	$6.0\ 10^{-6}$	0.7	0.3	0.3	90.8 ± 0.2
DIA	0.25–5	0.168	0.508	0.9992	1391.7 ± 0.1	36918 ± 0	$8.7\ 10^{-5}$	1.3	0.3	0.5	92.5 ± 0.3
DIHA	0.25–5	0.217	0.657	0.9987	21062.2 ± 0.1	48449 ± 0	$8.5\ 10^{-5}$	1.1	0.2	0.3	86.5 ± 0.2
HA	0.025–0.5	0.020	0.061	0.9989	5670.2 ± 0.0	456262 ± 0	$8.4\ 10^{-6}$	0.9	0.3	0.6	96.1 ± 0.3
HT	0.25–5	0.179	0.542	0.9991	5853.6 ± 0.1	4430 ± 0	$7.7\ 10^{-4}$	3.9	0.4	0.7	82.4 ± 0.9
Prometryn	0.025–0.5	0.018	0.053	0.9991	43721.4 ± 0.0	4009880 ± 0	$8.4\ 10^{-7}$	0.1	0.0	0.0	91.7 ± 1.0
Propazine	0.025–0.5	0.018	0.054	0.9991	9630.0 ± 0.0	1108789 ± 0	$3.1\ 10^{-6}$	1.1	0.1	0.2	87.7 ± 3.1
Simazine	0.025–0.5	0.019	0.057	0.9990	832.9 ± 0.0	24356 ± 0	$1.5\ 10^{-4}$	1.0	0.4	1.7	83.3 ± 0.1
Simetryn	0.025–0.5	0.010	0.032	0.9997	40018.7 ± 0.0	1674051 ± 0	$1.2\ 10^{-6}$	0.1	0.2	0.2	80.3 ± 0.2
Terbuthylazine	0.025–0.5	0.018	0.055	0.9991	22946.4 ± 0.0	1260632 ± 0	$2.8\ 10^{-6}$	2.7	0.1	0.2	92.2 ± 3.1
Terbutryn	0.025–0.5	0.008	0.023	0.9998	17886.2 ± 0.0	3042096 ± 0	$4.8\ 10^{-7}$	0.4	0.0	0.1	99.8 ± 0.2

[a] Confidence level 95%.
[b] 0.025 and 0.1 $\mu g L^{-1}$ for the nine triazines, DEA, DET and HA and 0.25 and 1 $\mu g L^{-1}$ for DEDIA, DEHA, DIA, DIHA and HT.
[c] 0.1 $\mu g L^{-1}$ for the nine triazines, DEA, DET and HA and 1 $\mu g L^{-1}$ for DEDIA, DEHA, DIA, DIHA and HT.

Acknowledgements

N. Rodríguez-González thanks the "Xunta de Galicia" and Campus do Mar (International Campus of Excellence) for the concession of her PhD. Grant and the Universidade da Coruña for the concession of her INDITEX-UDC 2015 Grant for her stay in Porto.

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ANEXO I-IV

Determination of triazine herbicides in seaweeds: development of a sample preparation method based on matrix solid phase dispersion and solid phase extraction clean-up. *Talanta* (2014) 121, 194-198.

Talanta 121 (2014) 194–198

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Determination of triazine herbicides in seaweeds: Development of a sample preparation method based on Matrix Solid Phase Dispersion and Solid Phase Extraction Clean-up

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ARTICLE INFO

Article history:
Received 18 September 2013
Received in revised form
20 December 2013
Accepted 28 December 2013
Available online 3 January 2014

Keywords:
Triazine herbicides
Seaweeds
Matrix Solid Phase Dispersion
Solid Phase Extraction
High Performance Liquid Chromatography
Diode Array Detection

ABSTRACT

A method using dual process columns of Matrix Solid Phase Dispersion (MSPD) and Solid Phase Extraction (SPE) has been developed for extracting and cleaning-up of nine triazine herbicides (ametryn, atrazine, cyanazine, prometryn, propazine, simazine, simetryn, terbuthylazine and terbutryn) in seaweed samples. Under optimized conditions, samples were blended with 2 g of octasilyl-derivatized silica (C_8) and transferred into an SPE cartridge containing ENVI^{TM}-Carb II/PSA (0.5/0.5 g) as a clean up co-sorbent. Then the dispersed sample was washed with 10 mL of *n*-hexane and triazines were eluted with 20 mL ethyl acetate and 5 mL acetonitrile. Finally the extract was concentrated to dryness, re-constituted with 1 mL methanol:water (1:1) and injected into the HPLC-DAD system. The linearity of the calibration curves was excellent in matrix matched standards, and yielded the coefficients of determination $\geq$ 0.995 for all the target analytes. The recoveries ranged from 75% to 100% with relative standard deviations lower than 7%. The achieved LOQs ($< 10\ \mu g\ kg^{-1}$) for all triazines under study permits to ensure proper determination at the maximum allowed residue levels set in the European Union Legislation. Samples of three seaweeds were subjected to the procedure proving the suitability of MSPD method for the analysis of triazines in different seaweeds samples.

1. Introduction

Seaweeds have been used since ancient times as food, fodder, fertilizer and as a source of medication. Nowadays, seaweeds are the raw material for industrial production of agar, carrageenan and alginates; however they still remain to be widely consumed as a source of food in Asian countries. Although in North America and Europe their use as food is more restricted, in recent years seaweeds have been increasingly recognized as healthy and attractive foods [1]. Edible seaweeds contain dietary fiber, high concentration of minerals, vitamins, proteins, polyunsaturated fatty acids and have a low content in saturated fats. On the other hand, seaweeds have also shown biological properties such as antibacterial, antiviral, antioxidant and antifungal [2,3]. Moreover, it has been reported that the chemical composition of seaweeds varies with species, habitats, maturity and environmental conditions [4].

Triazines, well-known herbicides, are applied to soil for the control of weeds in many agricultural crops, as well as railways, roadside and golf courses. The marine environment receives fluxes of these compounds mainly from agricultural origin. Their mechanism of action is via photosynthetic inhibition, and for this reason, they are only toxic for plants; however these compounds can affect the human health through the dietary intake. These compounds are highly persistent and can survive for many years in soil, water and organisms. Therefore, they are considered as an important class of chemical pollutants and atrazine and simazine have been included in the group of endocrine-disruptors by the Environmental Protection Agency of US [5]. As a result, the European Parliament and Council [6] concerning the residue levels in food and feed of plant and animal origins established the maximum permitted concentration in seaweed 0.01 and 0.05 mg kg^{-1} for simazine and terbuthylazine respectively. Moreover, a limit is not yet established for atrazine in seaweed, but its maximum permitted limit in edible vegetables is 0.05 mg kg^{-1}. For this reason, analytical methods for a rapid and sensitive determination of these compounds are required. However, seaweed is a complex matrix with different types of interfering compounds which make pesticide analysis difficult; in fact, studies of pesticides in seaweeds are limited and recent [7–9] and to the best of our knowledge there is only one reference in the literature devoted to the determination of triazines in seaweeds [10].

The most frequently used methodologies for the analysis of triazines in samples of vegetable and animal origins employ

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0039-9140/$ - see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.talanta.2013.12.063

solvent extraction procedures such as soxhlet [11,12], shake flask [13,14], sonication [10,13,15], microwave-assisted extraction (MAE) [11,12,16,17], and pressurized liquid extraction (PLE) [18,19]. Nevertheless, they generally need to add a clean-up step to decrease the presence of interferents in the final extract to reduce the detection limits of the methods and to avoid inaccurate results in the chromatographic determination, which is time consuming; many times it is expensive and simultaneously decreases the precision of the methodologies involved. Over the last years, different innovative procedures have been developed and applied for the determination of pollutants in complex matrices with improved capabilities, reduced clean up and concentration steps, the avoidance of toxic solvents and improved limits of detection. In this context, sorptive extraction techniques such as solid-phase extraction (SPE), dispersive solid phase extraction (dSPE), matrix solid-phase dispersion (MSPD), solid-phase micro-extraction (SPME) and stir bar sorptive extraction (SBSE) appear to be appropriate and they have been applied for analysis of triazines in different kinds of vegetation samples [20,21].

Matrix solid-phase dispersion is a sorptive extraction technique which involves the dispersion of the sample in a solid sorbent and subsequent elution with a relatively low solvent volume, allowing the simultaneous extraction and clean-up of analytes from solid samples [22]. If an additional clean up step is necessary, it is possible to use the MSPD column with another sorbent at its bottom. This technique shows a high flexibility and selectivity due to the variety of possible combinations of both sorbents and elution solvents [23]. Due to its simplicity and high throughput, MSPD methods have been developed for the extraction of different pesticides residues from different plants and plant materials [24]; however references for the determination of triazines by MSPD are still scarce and furthermore in most cases few triazines are included in these studies [25–28].

The aim of this work was the development and validation of an effective and simple method based on Matrix Solid Phase Dispersion (MSPD) and Solid Phase Extraction (SPE) Clean-up followed by High Performance Liquid Chromatography (HPLC) coupled to Diode Array Detection (DAD) for the simultaneous determination of nine triazine herbicides in seaweeds in order to be able to quantitate residues of these compounds in the range of the European maximum residue levels. Samples of three edible seaweeds were selected to illustrate the applicability of this method. To the best of our knowledge, no studies using MSPD have been done to extract these chemicals residues from seaweeds.

2. Experimental

2.1. Samples

Dried edible seaweeds. Sea lettuce (*Ulva Lactuca*), Wakame (*Undaria pinnatifida*), and Nori (*Porphyra umbilicalis*), from aquaculture production, were purchased from a local market in A Coruña city, NW, Spain. Samples were homogenized grounding them to a fine powder by an electric mill and stored in glass bottles out of light exposure until analysis.

2.2. Chemicals

(a) *Herbicide standards*. Herbicides (ametryn, atrazine, cyanazine, prometryn, propazine, simazine, simetryn, terbuthylazine and terbutryn) analytical standards were supplied by Sigma-Aldrich Inc. (St. Louis, MO, USA). The individual stock standard solutions of 1000 mg L^{-1} were prepared in methanol by exact weighing of high-purity substances and stored at −18 °C in dark. Then a mixture of all these compounds was prepared in methanol containing 10 mg L^{-1} each of individual triazine and stored at −18 °C. All working solutions were daily prepared by appropriate dilution of the 10 mg L^{-1} standard solutions with methanol:water (1:1) ratio.

(b) *Solvents*. *n*-hexane 95% and methanol were superpurity solvents obtained from Romil (Cambridge, UK). Acetonitrile (HPLC grade) and ethyl acetate (PAR, solvents for analysis of pesticide residues by GC) for instrumental analysis were obtained from Panreac (Barcelona, Spain). Milli-Q water was obtained from a purification system from Millipore (Billerica, MA).

(c) *Sorbents*. LC-8 Bulk packing and Supelclean™ ENVI-Carb II/PSA SPE Tube 6 ml (500 mg/500 mg) were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA).

(d) *Filters*. Polytetrafluoroethylene (PTFE) filters of 0.45 μm were obtained from Lida Manufacturing (Kenosha, WI, USA).

2.3. Materials and apparatus

A Visiprep® vacuum distribution manifold from Supelco (Bellefonte, PA, USA) was employed in the purification step. A Büchi R-3000 rotary evaporator (Büchi Labortechnic AG, Flawil, Switzerland) was used in the evaporation step.

Chromatographic analyses were carried out in a High Performance Liquid Chromatography-Diode Array Detector (HPLC–DAD). The system consisted of a 2695 pump with a 996 Diode Array Detector from Waters (Milford, MA, USA) and a computer running Empower 2 data processor. The column was a stainless steel column (150 mm × 4.6 mm ID, particle size 5 μm) packed with Hypersil GOLD C_{18} chemical bonded phase from Thermo Scientific (Austin, TX, USA).

2.4. Extraction procedure

The optimization study was carried out using a pesticide free sea lettuce seaweed sample spiked at the 1 mg kg^{-1} level. 1.0000 g of dried seaweed sample was homogenized with 2.00 g of LC-8 in a glass mortar with a pestle for 5 min. The final mixture was transferred into a 12-mL SPE cartridge containing a dual sorbent layer of 1 g Supelclean™ ENVI-Carb II/PSA (500/500 mg). Once packed, MSPD/SPE columns were connected to a Visiprep® vacuum distribution manifold and were washed with 10 mL of hexane. Elution was performed with 20 mL of ethyl acetate and 5 mL of acetonitrile (80:20) and the obtained eluate was evaporated to a drop in a rotary-evaporator and brought to dryness by a gentle nitrogen stream. The residue was reconstituted in 1 mL methanol:water (1:1) ratio and the solution was filtered through a 0.45 μm PTFE syringe filter.

2.5. HPLC-DAD conditions

The chromatographic analysis was carried out using the following ACN:H_2O gradient elution: ACN initial percentage of 30% (8 min) increased linearly to 40% in 5 min, increased to 50% in 5 min, after which the percentage was returned to the initial conditions in 9 min. A constant mobile phase flow rate of 1 mL min^{-1} and 20 μL of sample volume were used.

The absorbance was measured continuously in the 200–400 nm range and peak areas quantification was carried out at 222.7 nm in order to achieve maximum sensitivity. All triazine herbicides were identified initially by retention time and then by applying spectral contrast techniques (incorporated in Millenium^{32} software) the homogeneity of the spectral peak was confirmed. Finally a spectral identification was carried out contrasting the spectrum with a standard library created in the wavelength interval of 200–400 nm.

3. Results and discussion

3.1. MSPD optimization

In MSPD the analyte interacts with the solid support, the bonded phase and the dispersed matrix. For blending the sample, a glass mortar was used in order to avoid analyte losses that could occur with the use of materials such as porcelain [23]. In this technique the polarity of the sorbent and elution solvent are the key factors to achieve effectiveness of the extraction and purity of the final extract. Most methods reported have used reversed-phase materials bonded silica as the solid support. Theoretically, silica particles facilitate disruption of biological samples whereas the bonded alkyl chains contribute to dispersion and retention of lipophilic compounds [29]. C_8 and C_{18} are by far the most popular sorbents for analyte extraction from plant tissues; in this study C_8 was chosen as dispersant because it has been observed that C_8-bonded silica provided a more selective extraction with less co-elution compounds [30].

Although in some cases the MSPD extracts are clean enough to be directly subjected to instrumental analysis, a clean up step is often desirable. For this purpose an on-line clean-up step can be integrated into the sample preparation process by placing a layer of co-sorbent, obviously with different sorption behavior, at the bottom of MSPD cartridge. Based on our experience on clean up of animal feed extracts [31], solid phase extraction was carried out employing a dual-layer tube containing carbon/primary secondary amine (ENVI™-Carb II/PSA) sorbents separated by PE frit. ENVI-Carb II is a graphitized non-porous carbon that has a strong affinity towards planar molecules and can remove pigments (e.g., chlorophyll and carotenoids), and sterols. PSA is a polymerically bonded ethylenediamine-N-propyl phase that contains both primary and secondary amines, which retains fatty acids, organic acids, sugars and some polar pigments [32].

Selection of elution solvent is a function of analyte polarity, since the target analytes should be efficiently desorbed while the remaining components should be retained in the column. The generally employed solvents in the literature for elution of triazines in vegetable extracts from MSPD–SPE systems are acetonitrile [25], dichloromethane [27] and specially ethyl acetate [26,28,33]. In this study ethyl acetate and acetonitrile were tested; dichloromethane was avoided, according to principles of green chemistry, for being a chlorinated solvent.

The optimization of the MSPD–SPE procedure was carried out by spiking the sorbents with 1 mL of a standard solution containing 1 mg L^{-1} of each triazine. As ethyl acetate is the most common solvent for elution of triazines in MSPD procedures, the time consumed in the evaporation step is much lower with ethylacetate than with acetonitrile; hence preliminary assays were carried out with ethyl acetate. In order to determine solvent volume required for the complete elution of the target analytes, eluates of 5 mL were collected and the obtained results showed that at least 15 mL of ethyl acetate was necessary for recovering all compounds from the MSPD–SPE system. Recoveries obtained were higher than 90% for all compounds except for simetryn (70%), which although had a lower value than for the rest of triazines, it is acceptable in trace analysis. However, with a view for improving the recovery of simetryn, the sequence of 10 mL of ethyl acetate and 5 mL of acetonitrile was assayed and recoveries obtained were similar to those achieved with ethyl acetate for most of compounds but recovery of simetryn increased until 86%. Therefore the combination of 10 mL ethyl acetate + 5 mL acetonitrile was selected as elution solvent.

The selected conditions were applied to samples of sea lettuce, which did not contain triazine residues at detectable concentrations. For this purpose, 1.0000 g of dried seaweed was spiked at the 1 mg kg^{-1} level (equivalent to 0.08 mg kg^{-1} of fresh tissue) and subjected to the procedure previously optimized with standards. Recoveries obtained for spiked seaweed were between 50% and 65% for all analytes which implies a strong interaction of all studied compounds with the matrix. Therefore additional volumes of ethyl acetate and/or acetonitrile were collected but very unsatisfactory recoveries were obtained in all cases, and only the tandem of 20 mL ethyl acetate and 5 mL acetonitrile achieved recoveries higher than 60% for all compounds. Several authors have pointed out that washing the column with a solvent, such as hexane or water, prior to elution of the target analytes can have a huge influence on the performance of the MSPD method [29,30]. Therefore an experiment on rinsing the sample with 10 mL of hexane, prior to analytes elution with 20 mL ethyl acetate and 5 mL acetonitrile, was carried out. With this procedure, schematized in Fig. 1, satisfactory recoveries were obtained for all triazines (up to 80%). As an illustration of the results obtained, Fig. 2 shows the chromatograms corresponding to unspiked and spiked sea lettuce extracted and purified under the procedure considered.

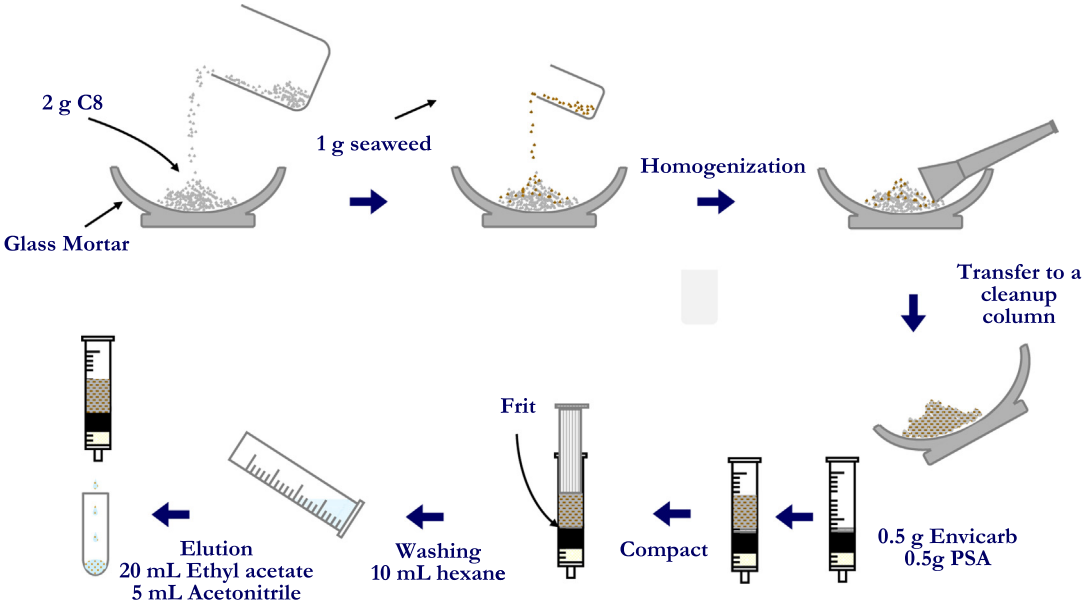

Fig. 1. Scheme of the final conditions of MSPD–SPE procedure.

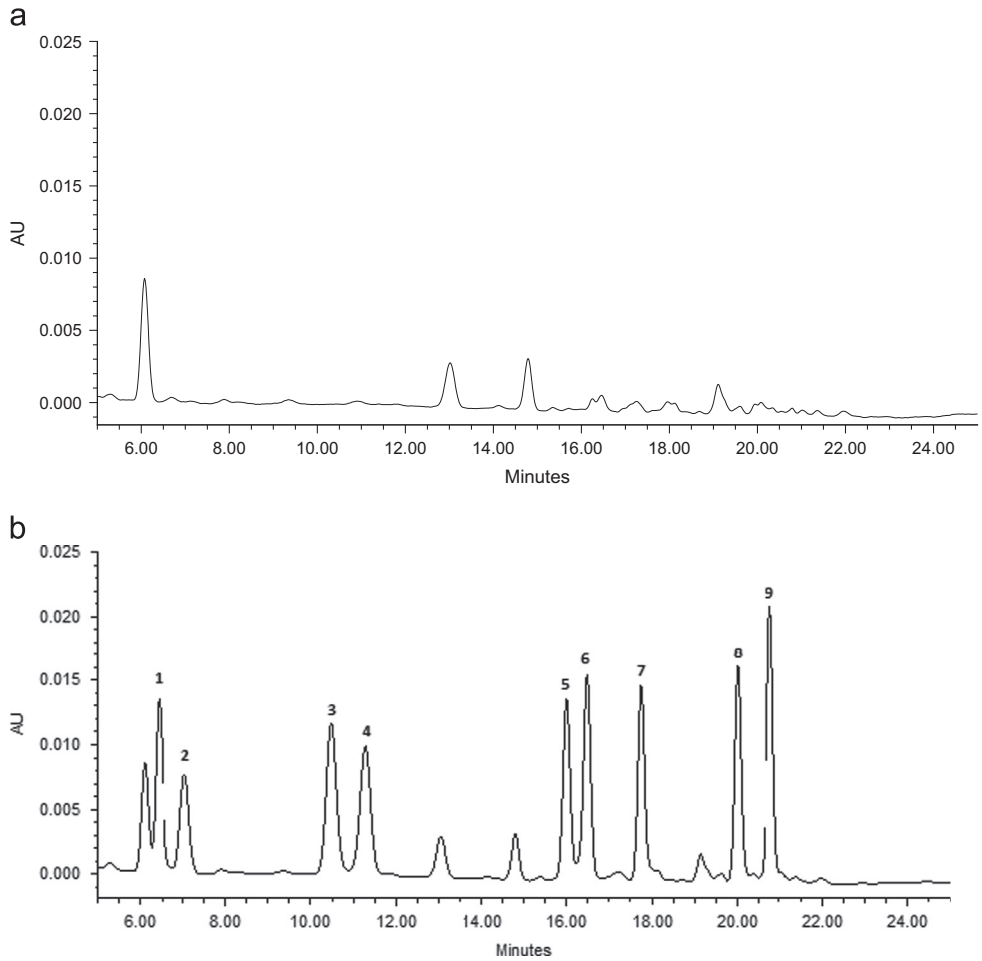

Fig. 2. (a) MSPD sea lettuce extract chromatogram and (b) MSPD spiked sea lettuce extract chromatogram. Target compounds are numbered as follows: (1) simazine, (2) cyanazine, (3) simetryn, (4) atrazine, (5) ametryn, (6) propazine, (7) terbuthylazine, (8) prometryn, and (9) terbutryn.

3.2. Method validation

The method was validated in terms of linearity, recoveries, precision and limits of detection and quantification. The validation data are presented in Table 1.

All quantitative results were calculated using matrix matched standards prepared by spiking the final extracts from un-spiked samples of sea lettuce with different levels of triazines as recommended by the European guidelines [34]. Good linearity of the calibration curves was obtained for all compounds over the whole range (0.1–2 mg kg^{-1} dried sample) evaluated by duplicate analysis at five different concentration levels. The target compounds showed coefficients of determination (R^2) higher than 0.9992 for all triazines except for prometrin and terbutryn which showed only slightly lower R^2 ($>$ 0.995).

The matrix effect was evaluated since signal suppression or enhancement can severely compromise quantitative analysis of the compounds at trace levels, and therefore can greatly affect the method reproducibility and accuracy. The matrix effect was studied by comparison of the slopes of the calibration curves in solvent and in the extract obtained after the MSPD procedure. Both the solvent and the matrix calibration curves had good linearity, with determination coefficients higher than 0.9999 for solvent calibration curves and 0.9952 for matrix matched ones. All compounds showed a strong matrix effect since the deviation of the matrix calibration slope from the solvent calibration slope was higher than 20% (between 29% and 42%), which indicated the need of using matrix matched standards for quantification purposes.

Table 1
Validation data for the MSPD-HPLC-DAD method obtained employing sea lettuce seaweed.

Compound	LOD[a] (μg kg^{-1})	LOQ[a] (μg kg^{-1})	Correlation coefficient (R^2)	Recovery ± RSD (%) ($n=5$)	
				0.1 mg kg^{-1}	**1 mg kg^{-1}**
Simazine	3.6	6.5	0.9997	81.2 ± 6.3	88.2 ± 0.9
Cyanazine	3.0	7.3	0.9998	80.2 ± 5.7	79.5 ± 0.6
Simetryn	3.8	6.2	0.9999	80.8 ± 5.3	83.0 ± 2.5
Atrazine	2.7	5.3	0.9992	85.9 ± 5.5	81.0 ± 2.8
Ametryn	2.9	5.9	0.9997	90.8 ± 7.3	83.0 ± 1.0
Propazine	3.6	6.0	0.9992	91.7 ± 5.1	91.8 ± 0.6
Terbuthylazine	1.5	4.3	0.9996	76.0 ± 7.4	84.8 ± 1.7
Prometryn	1.4	4.1	0.9973	82.8 ± 7.2	96.6 ± 2.8
Terbutryn	1.6	4.5	0.9952	86.1 ± 3.6	83.2 ± 1.1

[a] Results expressed in μg kg^{-1} fresh sample.

The accuracy and precision of the proposed method were investigated by analysis of five replicates of uncontaminated sea lettuce samples spiked at two different concentration levels (1 and 0.1 mg kg^{-1} dried sample, equivalent to 0.08 and 0.008 mg kg^{-1} fresh sample respectively). The lowest spiked concentration was selected to test the method performance at the recent restrictive residue level set by European Union Legislation for simazine in edible seaweeds [35]. The obtained results demonstrated that the method achieved satisfactory recoveries in the range of 75–100% in all cases, with associate standard deviations below 7% for all compounds,

Table 2
Mean recoveries and RSD values of triazines in wakame and nori samples ($n=5$) spiked at two levels applying the MSPD-HPLC-DAD method.

Compound	Wakame		Nori	
	0.1 mg kg^{-1}	1 mg kg^{-1}	0.1 mg kg^{-1}	1 mg kg^{-1}
	Recovery ± RSD (%) ($n=5$)			
Simazine	98.7 ± 4.7	90.4 ± 4.1	83.6 ± 6.7	85.9 ± 1.5
Cyanazine	87.0 ± 3.4	85.7 ± 1.2	64.8 ± 8.8	87.5 ± 1.9
Simetryn	94.2 ± 4.5	84.6 ± 0.3	65.1 ± 3.0	85.5 ± 1.4
Atrazine	92.3 ± 0.5	89.1 ± 1.4	78.6 ± 1.5	89.1 ± 1.7
Ametryn	102.1 ± 3.9	89.6 ± 1.2	77.5 ± 2.2	91.0 ± 0.8
Propazine	92.9 ± 6.8	90.5 ± 1.5	64.6 ± 3.3	90.5 ± 1.2
Terbuthylazine	86.0 ± 1.0	90.8 ± 1.4	70.4 ± 2.4	86.7 ± 1.4
Prometryn	120.2 ± 3.1	91.0 ± 1.8	70.9 ± 2.8	86.8 ± 0.7
Terbutryn	99.1 ± 0.7	89.4 ± 1.8	64.7 ± 5.2	85.4 ± 1.0

which are in the acceptance range [34]. The obtained recoveries and relative standard deviation are comparable or even better than those provided by other authors for the determination of some of these pollutants by MSPD in fruits and vegetables [27,28,33].

The limits of detection (LODs) and limits of quantification (LOQs) were calculated as the minimum amount of target analyte that led to a chromatogram peak with a signal-to-noise ratio of 3 and 10 respectively, determined experimentally from fortified samples [34]. As can be seen in Table 1, the LODs varied from 1.4 to 3.8 μg kg^{-1} and LOQs values varied from 4.1 to 7.3 μg kg^{-1} in fresh sample. Therefore, the obtained LODs and LOQs were satisfactory and allowed the determination of these compounds at the levels required by the legislation of seaweed for human consumption. By comparing the LODs obtained by the proposed methodology with those reported in the literature using MSPD in horticultural matrices, better sensitivity is attained [27,28,33].

Reliability of the method was evaluated in terms of recovery by spiking two edible seaweed samples: a red one (nori) and a brown one (wakame) at a concentration level of 1 and 0.1 mg kg^{-1} dried samples. The analytical recoveries, calculated using matrix matched standards, obtained for five replicates ($n=5$) of the samples spiked with the triazine herbicides are presented in Table 2. As can be seen the recovery values obtained for wakame ranged between 85% and 100% for all compounds, except for prometrin at the low level (120%), with RSD lower than 7%. In the case of nori recoveries fluctuated from 85% to 90% with RSD values below 2% at the high level whereas they were between 65% and 85% with RSD below 9% at the low level. Therefore we can conclude that this method could be established as a suitable method for routine analysis to screen trace levels in different types of seaweed in compliance with EU directives.

4. Conclusion

A procedure for the analysis of nine triazines from seaweed samples based on MSPD and SPE has been developed. The method uses C_8 as dispersant with ENVI-Carb™/PSA co-column and *n*-hexane as washing solvent followed by a combination of 20 mL ethyl acetate and 5 mL acetonitrile as elution solvent. The developed method provides satisfactory accuracy and precision for the determination of triazines in seaweed with LODs and LOQs adequate to carry out analysis of samples in the concentrations required by the European Union regulations. The method was successfully applied to the analysis of three seaweed samples (sea lettuce, wakame and nori). The main advantages of this methodology when compared with conventional methods of sample preparation to screen triazines in vegetable matrices are easy of work-up, fast, low cost, avoidance of clean-up procedure, as well as the significant reduction of organic solvents in agreement with the principles of the Green Chemistry.

Acknowledgments

The authors acknowledge the financial support of the "Ministerio de Educación y Ciencia" (Project CTQ2007-63949/BQU) and the "Xunta de Galicia" (Projects 09MDS038103PR and 2010/52). N. Rodríguez-González also thanks the "Xunta de Galicia" for the concession of her award under the program "Campus del Mar".

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ANEXO I-V

Development of a matrix solid phase dispersion methodology for the determination of triazine herbicides in mussels. *Food Chemistry* (2015) 173, 391-396.

Food Chemistry 173 (2015) 391–396

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Development of a Matrix Solid Phase Dispersion methodology for the determination of triazine herbicides in mussels

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ARTICLE INFO

Article history:
Received 30 May 2014
Received in revised form 17 September 2014
Accepted 21 September 2014
Available online 7 October 2014

Chemical compounds studied in this article:
Ametryn (PubChem CID: 13263)
Atrazine (PubChem CID: 2256)
Cyanazine (PubChem CID: 30773)
Prometryn (PubChem CID: 4929)
Propazine (PubChem CID: 4937)
Simazine (PubChem CID: 5216)
Simetryn (PubChem CID: 13905)
Terbuthylazine (PubChem CID: 22206)
Terbutryn (PubChem CID: 13450)

Keywords:
Triazines
Mussel
Matrix Solid Phase Dispersion
High performance liquid chromatography
Diode array detection

ABSTRACT

A method based on Matrix Solid Phase Dispersion (MSPD) for determination of nine triazines in mussels has been optimised in terms of the sorbents used for extracting and cleaning-up. Two dispersing agents: C_{18} and florisil, and eight cleanup co-sorbents: florisil, silica, silica/alumina, Envi™ Carb, Envi-Carb-II/PSA, SAX/PSA, Envi-Carb-II /SAX/PSA and C_{18} were assayed. Analytes were eluted using 20 mL of ethyl acetate and 5 mL of acetonitrile and finally the extract was concentrated to dryness, re-constituted with 1 mL methanol and determined by HPLC–DAD. The best results were obtained with C_{18} as dispersing agent and Envi-Carb-II/SAX/PSA as clean-up co-column. Recoveries ranged between 79% and 99% and repeatability and reproducibility were below than 16% for all compounds. The linearity of the calibration curves yielded the $R^2 \geqslant 0.9993$. The LOQ values ranged from 0.10 to 0.18 mg kg^{-1} dried sample. Finally the method was applied to the analysis of mussel samples from Galicia (NW Spain).

1. Introduction

The accumulation of environmental chemical contaminants in fish and shellfish pose a potential human health hazard. Coastal areas are subject to strong anthropogenic pressures as a consequence of their location between land and sea and they also receive fluxes of pesticides mainly of agricultural origin (Carafa et al., 2007; Fernández, Fernández- Boán, Verísimo, & Freire, 2013). Impacts of herbicides in the aquatic ecosystem are both direct and indirect, spanning from instantaneous effects to long-term effects as bioaccumulation and biomagnification through the food chain (Carafa et al., 2007).

Triazines are a group of herbicides that are present in the ten most-used herbicide formulations in Europe. Triazines have been used extensively as herbicides to provide pre- and post-emergence of grasses, crops and many weeds in cereals (Wang et al., 2010). Because of their relatively low soil adsorption and high solubility in water, these compounds migrate from soil to water (Andreu & Picó, 2004) and due to the water cycle, fluxes of them reach seawater and affect the marine biota, having the coastal systems little capacity to degrade these compounds (Fernández et al., 2013). Therefore, they are considered as an important class of chemical pollutants and atrazine and simazine have been included in the Endocrine Disruption Screening Program by the U.S. Environmental Protection Agency (2009).

As a result, the European Union has also included simazine and atrazine in the list of 33 priority substances in the EU Water Framework Directive (2000/60/EC) by way of Decision 2455/2001/EC. Recently, terbutryn has been added to the list of priority

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http://dx.doi.org/10.1016/j.foodchem.2014.09.153
0308-8146/

substances by way of Commission Proposal 2011/876/EC. Moreover, the Directive 2008/105/EC sets the Environmental Quality Standards (EQS) for these compounds in water and also shows the need to set EQS for these compounds in sediments and/or biota. Although limit values have not established yet by European Union for triazines in fish and fishery products, the U.S. Food and Drug Administration (FDA) has set a tolerance level of 12 mg kg^{-1} ww for simazine in fish (2011).

Aquatic organisms, such as fish and shellfish, are able to accumulate pesticide residues in much higher concentration than the surrounding water (LeDoux, 2011). Thus, monitoring their content in seafood is necessary to estimate human exposure to these substances. Bivalves are suitable organisms for using in environmental monitoring programs due to their wide geographical distribution, great sensitivity to environmental pollutants and high accumulation rate; furthermore they are stationary and are normally the dominant species in their habitat (Binelli, Ricciardi, Riva., & Provini, 2006).

The more frequently used methodologies for the analysis of triazines in samples of animal origin employ solvent extraction procedures such as soxhlet (Rosenblum, Hieber, & Morgan, 2001), shake flask (Baranowska, Barchanska, Abuknesha, Price, & Stalmach, 2008; Tsuda, Nakamura, Inoue, & Tanaka, 2009), sonication (Carafa et al., 2007; Salvadó, Quintana, & Hidalgo, 2006), microwave-assisted extraction (MAE) (Cheng et al., 2007; Fernández et al., 2013) and pressurised liquid extraction (PLE) (Damásio et al., 2010; Köck et al., 2010). Nevertheless, they generally need to add a clean-up step to decrease the presence of interferents in the final extract to reduce the detection limits of the methods and to avoid inaccurate results in the chromatographic determination. In the last years, different innovation procedures have been developed and applied for the determination of pollutants in complex matrices with improved capabilities, reduced clean up and concentration steps, the avoidance of toxic solvents and improved limits of detection. In this context, modern extraction techniques such as dispersive solid phase extraction (dSPE), matrix solid-phase dispersion (MSPD), solid-phase micro-extraction (SPME) and stir bar sorptive extraction (SBSE) appear to be appropriate (Beceiro-González, González-Castro, Muniategui-Lorenzo, López-Mahía, & Prada-Rodríguez, 2012). The main difference among these techniques is that the retention in the two latter techniques is based on the distribution equilibrium between the sample matrix and a non-miscible sorbent phase.

MSPD is an extraction procedure which combines aspects of several analytical techniques allowing sample homogenisation, disruption, extraction, fractionation and clean-up within a single process (Barker, 2000). In MSDP the solid sample is blended in a mortar with an appropriate sorbent to obtain complete disruption and dispersion of the sample on the solid support. The blend is packed into a column from which the analytes are eluted with a relatively low solvent volume (Ramos, González, & Ramos, 2009). Often, a co-sorbent material is placed at the bottom of the column to be filled with the blended sample to assist the extract clean up (Capriotti, Cavaliere, Lagana, Piovesana, & Samperi, 2013). The key factors for the success of MSPD are its feasibility, flexibility, versatility, high throughput, low cost and rapidity. MSPD methods have been developed for the extraction of triazine residues from different plants and plant materials (Ramos et al., 2009; Rodríguez-González, González-Castro, Beceiro-González, Muniategui-Lorenzo, & Prada-Rodríguez, 2014; Wen et al., 2012); however references for the determination of triazines in animal tissues by MSPD are scarce. As far as we know, there are only two references in the literature devoted to the determination of triazines in fish by MSPD and furthermore few triazines are included in these studies (Gaunt & Barker, 2000; Souza-Caldas et al., 2013).

The aim of this work was the development and validation of an effective, simple and fast method for the simultaneous determination of nine triazine herbicides in mussels based on Matrix Solid Phase Dispersion (MSPD) and Solid Phase Extraction (SPE) clean-up followed by High performance liquid chromatography (HPLC) coupled to Diode array detection (DAD). To the best of our knowledge, no studies using MSPD have been done to extract these chemicals residues from shellfish. As an application, the analysis of mussel samples from Galicia (NW Spain) was carried out. Galicia is the highest producer of mussels in Europe and the fish and shellfish industry is very important in this area, exporting their products around the world. Therefore the study of levels of these contaminants on fish and fishery products is of a great economic and environmental importance for Galicia.

2. Materials and methods

2.1. Samples

Mussels (*Mytilus galloprovincialis*) were purchased from a local market in A Coruña city (Northwest of Spain). Mussels were removed from the shell, homogenised and freeze-dried. Then samples were homogenised again grounding it to a fine powder by an electrical mill and finally they were stored in glass bottles out of light exposure until analysis.

2.2. Chemicals

(a) *Herbicide standards* – Herbicides (ametryn, atrazine, cyanazine, prometryn, propazine, simazine, simetryn, terbuthylazine and terbutryn) analytical standards were supplied by Sigma–Aldrich (Inc. St. Louis, MO, USA). The individual stock standard solutions of 1000 mg L^{-1} were prepared in methanol by exact weighing of high-purity substances and stored at −18 °C in the dark. Then a mixture of all the compounds was prepared in methanol containing 10 mg L^{-1} each individual triazine and stored at −18 °C. All working solutions were daily prepared by appropriate dilution of the 10 mg L^{-1} standard solutions with methanol.

(b) *Solvents* – n-hexane 95% and methanol were superpurity Solvents from Romil (Cambridge, UK). Acetonitrile (ACN) (HPLC grade) and ethyl acetate (PAR, solvents for analysis of pesticide residues by GC) for instrumental analysis were from Panreac (Barcelona, Spain). Milli-Q water was obtained from a purification system from Millipore (Billerica, MA).

(c) *Sorbents – bulk packing:* Envi-18 and LC-Florisil were from Sigma–Aldrich (Inc. St Louis, MO, USA). *SPE tubes*: Envi-Florisil (1 g and 2 g), LC-Silica (1 g), LC-Silica/Alumina (1 g/1 g), Envi™ Carb (1 g), Envi-Carb-II/PSA (500 mg/500 mg), SAX/PSA (500 mg/500 mg), Envi-Carb II/SAX/PSA SPE (500 mg/500 mg/500 mg) were from Sigma–Aldrich (Inc. St Louis, MO, USA); C_{18} SepPak Plus was from Waters (Milford, MA, USA).

(d) *Filters* – Polytetrafluoroethylene (PTFE) filters of 0.45 μm were from Teknocroma (Barcelona, Spain).

2.3. Materials and apparatus

A Visiprep® vacuum distribution manifold from Supelco (Bellefonte, PA, USA) was employed in the purification step. A Büchi R-3000 rotary evaporator (Büchi Labortechnic AG, Flawil, Switzerland) was used in the evaporation step.

Chromatographic analyses were carried out in a high performance liquid chromatography-diode array detector (HPLC–DAD). The system consisted of a 2695 pump with a 996 Diode Array Detector from Waters (Milford, MA, USA). The column was a stainless steel column (150 mm × 4.6 mm ID, particle size 5 μm) packed

with Hypersil GOLD C_{18} chemical bonded phase from Thermo Scientific (Austin, TX, USA).

2.4. Extraction procedure

Optimisation experiments of the several sorbents tested were carried out by analysing a triazine-free freeze-dried mussel sample spiked with the triazines to obtain a final concentration of each analyte in the mussel of 2 mg kg^{-1}. Under final working conditions, 0.5 g of freeze-dried mussel sample was homogenised with 2 g of Envi-C_{18} in a glass mortar with a pestle for 5 min. The final mixture was transferred into a 20-mL SPE cartridge containing a triple sorbent layer of 1.5 g Supelclean™ Envi-Carb-II/SAX/PSA (500/500/500 mg). Once packed, MSPD/SPE columns were connected to a Visiprep® vacuum distribution manifold and washed with 10 mL of hexane. Elution was performed with 20 mL of ethyl acetate and 5 mL of ACN and the obtained eluate was evaporated to a drop in rotary-evaporator and got to dryness by a gentle nitrogen stream. The residue was reconstituted in 1 mL methanol and the solution was filtered through of a 0.45 μm syringe filter of PTFE.

2.5. HPLC–DAD conditions

The chromatographic analysis was carried out using the following ACN:H_2O gradient elution: ACN initial percentage of 30% (8 min), increased linearly to 40% in 5 min; increased to 50% in 5 min, after which the percentage was returned to the initial conditions in 9 min. A constant mobile phase flow rate of 1 mL min^{-1} and 20 μL of sample volume were used.

The absorbance was measured continuously in the 200-400 nm range and peaks areas quantification were carried out at 222.7 nm in order to achieve maximum sensitivity. All triazine herbicides were identified initially by retention time and then by spectral identification contrasting the spectrum with a standard library created in the same wavelength interval.

3. Results and discussion

3.1. MSPD optimisation

The effectiveness of an MSPD procedure depends on the sorbent/solvent combination. The following parameters were taking into consideration in the univariate optimisation of the MSPD procedure: type and amount of dispersing agent and sorbents used for the clean-up. Most methods reported have used reversed-phase materials, such as C8 and C_{18}-bonded silica as the solid support, whereas normal phase such as florisil or silica are used less frequently (Kristenson, Ramos, & Brinkman, 2006). The lipophilic character of the reversed phase material is believed to facilitate disruption, dispersion and retention of lipophilic compounds whereas normal phase materials interact with sample components only by adsorption and, obviously, are not able to dissolve the sample matrix (García-López, Canosa, & Rodríguez, 2008). Although in some cases the MSPD extracts are clean enough to be directly subjected to instrumental analysis, a clean-up step is often desirable. For this purpose an on-line clean-up step can be integrated into the sample preparation process by placing a layer of co-sorbent with different sorption behaviour, at the bottom of MSPD cartridge.

For optimisation of the MSPD procedure 0.5 g of pesticide free mussel sample was spiked at the 2 mg kg^{-1} level. A reversed phase (C_{18}) and a normal phase (florisil) sorbents were tested as dispersants (1.5 g each). C_{18} was chosen because it is by far the most popular sorbent, especially for analyte extraction from animal tissues (Kristenson et al., 2006) because cell membranes could be disrupted through the solubilisation of phospholipid components in C_{18} material. Florisil was selected because it has been reported as the more efficient normal phase material in terms of lipid retention from fish and shellfish tissues (Negreira, Rodríguez, Rodil, Rubí, & Cela, 2013).

First assays were carried out with florisil because the combination of normal phase as dispersant and reversed phase as clean up co-sorbent has been reported as more efficient in terms of lipids retention in fish (Canosa, Rodríguez, Rubí, Ramil, & Cela, 2008). For this purpose, florisil (1.5 g) as dispersant was assayed with Envi-Carb (1 g) and C_{18} (1 g) as co-sorbents. Based on our experience, elution of the cartridges was performed with 20 mL of ethyl acetate and 5 mL of acetonitrile after rinsing with 10 mL of hexane (Rodríguez-González et al., 2014). The extracts obtained using C_{18} were very dark, indicating coelution of matrix interferences and were not analysed. Envi-Carb provided extracts clean enough to be injected, however recoveries obtained ranged between 29% and 69%, and therefore the use of florisil as dispersant was discarded.

Considering C_{18} (1.5 g) as dispersant the following co-sorbents were assayed: florisil (1 g and 2 g), silica (1 g), silica and alumina (1 g/1 g), Envi-Carb-II/PSA (0.5 g/0.5 g), SAX/PSA (0.5 g/0.5 g), and Envi-Carb-II/SAX/PSA (0.5 g/0.5 g/0.5 g). Experiments carried out using florisil (1 g) and silica (1 g) provided extracts very dark and with solid particles and were not analysed further. The use of SAX/PSA (0.5 g/0.5 g), and especially silica/alumina (1 g/1 g) and florisil (2 g) led to intensely coloured extracts; however they could be processed because the filtration through PTFE filters considerably decreased the colour of the extracts. Envi-Carb-II/PSA (0.5 g/0.5 g) and Envi-Carb-II/SAX/PSA (0.5 g/0.5 g/0.5 g) provided extracts clean enough before filtration through PTFE filters, which indicates the importance of using graphitized carbon black on the cleaning of mussel extracts.

Recoveries obtained with florisil and silica/alumina were lower than 80% for all compounds, while the use of SAX/PSA yielded recoveries ranging from 72% to 89%. Satisfactory recoveries were achieved with systems based on carbon, Envi-Carb-II/PSA and Envi-Carb-II/SAX/PSA, with values ranging from 79% to 96% for the first system and from 85% to 102% for the second one (Fig. 1). As well as recoveries, taking into account the higher deviation of the results in the case of Envi-Carb-II/PSA when comparing with Envi-Carb-II/SAX/PSA, the system Envi-Carb-II/SAX/PSA was chosen as clean up co-sorbent. Envi-Carb-II is a graphitized non porous carbon that has a strong affinity towards planar molecules and can remove pigments (e.g., carotenoids), and sterols. In addition, PSA is a polymerically bonded ethylenediamine-N-propyl phase that contains both primary and secondary amines, that retains fatty acids, organic acids, sugars and some polar pigments. SAX is a quaternary amine that offers additional ion exchange capacity for removing matrix components providing low UV and MS extractables for lower background and greater sensitivity.

In developing the method, it was observed that after several injections ghost peaks appeared. After checking the instrument and restoring the column performance by running a strong solvent, the ghost peaks were attributable to a late eluting compound present in sample. Therefore the sample preparation method was checked, in terms of recovery, increasing the amount of dispersant to 2 g and the problem of ghost peaks was resolved. Thus, 2 g of C_{18} as dispersant and Envi-Carb-II/SAX/PSA (0.5 g/0.5 g/0.5 g) as clean-up co-sorbents were selected for the whole procedure. Fig. 2 shows the chromatograms corresponding to unspiked and spiked mussel sample extracted and purified under the considered procedure.

3.2. Method validation

The method was validated in terms of linearity, accuracy, precision and limits of detection and quantitation according to "valida-

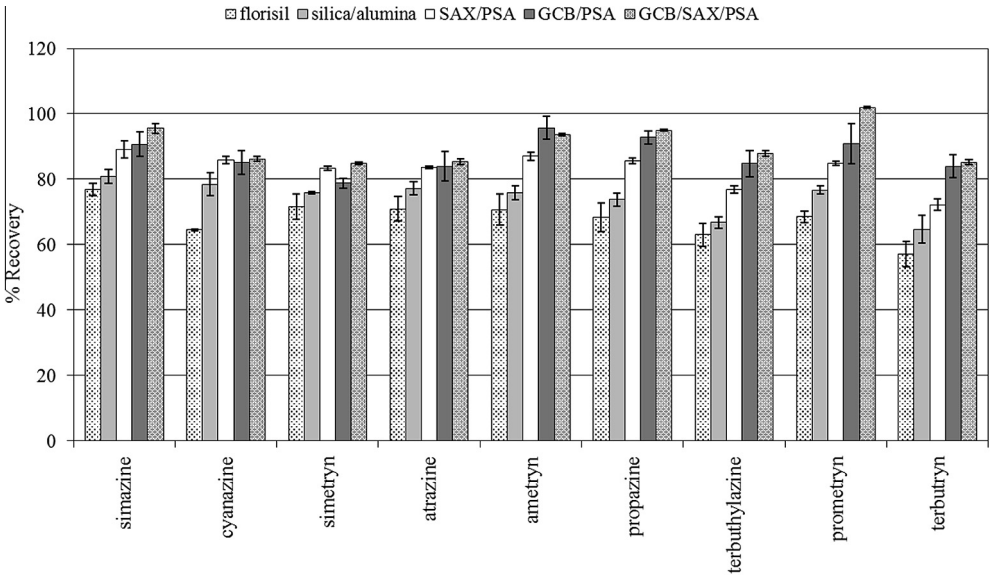

Fig. 1. Analytical recoveries of triazines from mussel samples using C_{18} as dispersant with different cleanup co-sorbents.

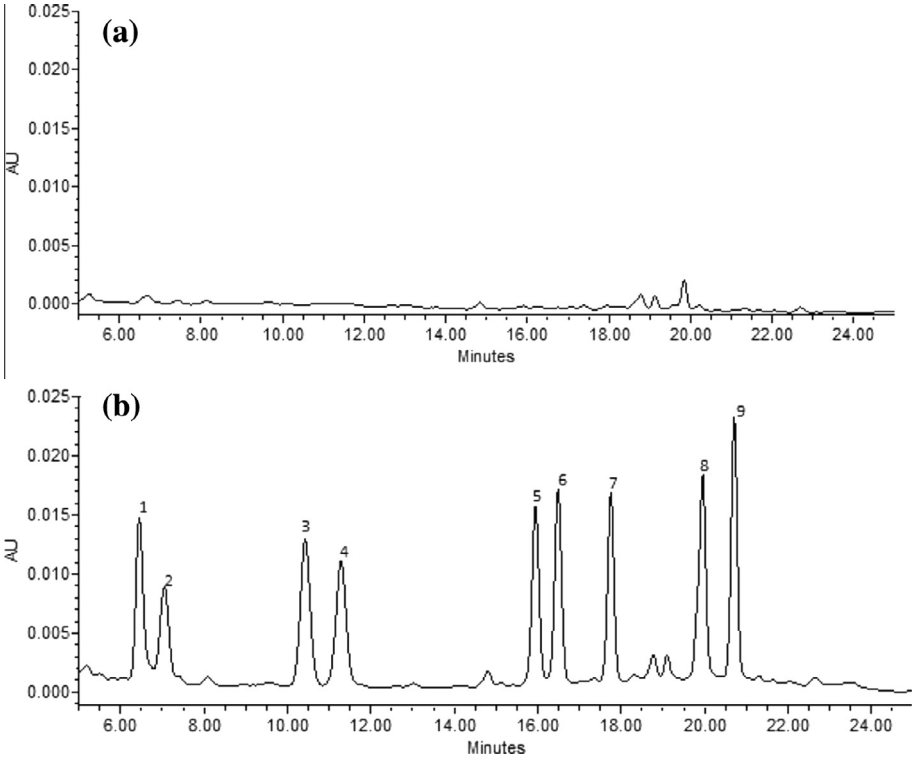

Fig. 2. (a) MSPD mussel extract chromatogram and (b) MSPD spiked mussel extract chromatogram. Target compounds are numbered as follows: (1) simazine, (2) cyanazine, (3) simetryn, (4) atrazine, (5) ametryn, (6) propazine, (7) terbuthylazine, (8) prometryn, (9) terbutryn.

tion parameters and criteria" from SANCO Guidelines for method validation and quality control procedures for pesticide residue analysis in food and feed (Directorate of General Health and Consumer Protection. Document n° SANCO/12571, 2013). The validation data are presented in Tables 1 and 2.

Quantitative results were calculated using matrix matched standards prepared by spiking the final extracts from blank samples of mussels with different levels of triazines. The linearity of the calibration curves was calculated at a concentration range between 0.2–4 mg kg^{-1} dried sample by duplicate analysis at six different concentration levels. As can be seen in Table 1 excellent

Table 1
Coefficient determination (R^2) and LODs and LOQs.

Compound	Determination coefficient (R^2)	LOD (mg kg^{-1})	LOQ (mg kg^{-1})
Simazine	0.9999	0.050	0.13
Cyanazine	0.9998	0.068	0.18
Simetryn	0.9993	0.038	0.10
Atrazine	0.9993	0.043	0.12
Ametryn	0.9996	0.045	0.12
Propazine	0.9993	0.041	0.11
Terbuthylazine	0.9996	0.046	0.12
Prometryn	0.9993	0.049	0.13
Terbutryn	0.9997	0.036	0.10

Table 2
Precision and accuracy of target compounds in mussel samples with MSDP–HPLC–DAD.

Compound	Repeatability (%RSD, $n = 5$)		Reproducibility (%RSD, $n = 6$)		Accuracy (%) recovery ($n = 5$)	
	0.2 mg kg^{-1}	2 mg kg^{-1}	0.2 mg kg^{-1}	2 mg kg^{-1}	0.2 mg kg^{-1}	2 mg kg^{-1}
Simazine	1.5	1.6	7.5	2.8	99	97
Cyanazine	10	1.9	12	2.3	96	95
Simetryn	3.6	2.9	4.4	3.9	79	93
Atrazine	0.6	0.5	2.3	2.5	82	96
Ametryn	5.1	2.2	5.9	3.1	86	93
Propazine	5.6	2.1	9.0	2.6	84	94
Terbuthylazine	4.1	1.9	5.6	2.4	86	95
Prometryn	3.1	1.6	16	2.6	84	94
Terbutryn	3.8	2.6	8.0	2.7	89	91

linearities were obtained with coefficients of determination (R^2) higher than 0.9993 for all triazines.

The limits of detection (LODs) and quantitation (LOQs), calculated as the minimum amount of target analyte that led to a chromatogram peak with a signal-to-noise ratio of 3 and 10, respectively, are shown in Table 1. The LODs varied from 0.036 to 0.068 mg kg^{-1} and LOQs from 0.10 to 0.18 mg kg^{-1}, referred to the freeze-dried sample. The reduction in the weight of mussel samples during freeze-drying accounted for ca. 80% (0.5 g of freeze-dried sample equivalent to ca. 2.5 g of fresh tissue); therefore, approximately 5-fold lower LOQ values are achieved when referred to fresh sample. By comparing the LOQs obtained by the proposed methodology with those reported in the literature using MSPD in fish, better sensitivity is attained (Gaunt & Barker, 2000; Souza-Caldas et al., 2013).

Precision and accuracy of the proposed method were determined by spiking 0.5 g of uncontaminated mussel sample at two concentration levels (0.2 y 2 mg kg^{-1}). The accuracy of the method was evaluated regarding the recovery assay by analysis of five replicates at each fortification level. The obtained results demonstrated that the method achieved satisfactory recoveries for all compounds in the range of 79–99% for the low level and of 91–97% for the high level (Table 2), which indicate that the method meets the requirements stipulated (DG SANCO/12571/2013). The precision of the method, expressed as the relative standard deviation (RSD), was evaluated measuring five replicates samples at the same day (intraday precision) and six replicate samples on three consecutive days (inter-day precision). RSD values lower than 10% and 16% for intraday and interday precision respectively were obtained (Table 2), which are in the acceptance range of the DG SANCO/12571/2013 of the European Quality Control Guidelines (RSD < 20%). It is worth noting that the obtained recoveries and relative standard deviation are comparable or even better to those provided by other authors for the determination of some of these pollutants by MSPD in fish (Gaunt & Barker, 2000; Souza-Caldas et al., 2013).

Finally, the developed method was applied to analyse the different target analytes in five mussel samples purchased from local markets of A Coruña (NW Spain). Although none of them contained detectable amount of target pesticides, studies regarding the presence of these compounds are required on shellfish harvested from aquaculture ponds because these areas are subject to shore side contaminant discharges.

4. Conclusions

The suitability of a procedure based on MSPD and SPE for the extraction of nine triazines from shellfish samples has been demonstrated for the first time. The method uses C_{18} as dispersant with Envi-Carb-II/SAX/PSA co-column followed by a combination of 20 mL ethyl acetate and 5 mL acetonitrile as elution solvent. The developed method provides satisfactory accuracy and precision for the determination of triazines in mussels. The main advantages of this methodology when compared with classical methods of sample preparation to determine triazines in animal tissues are easy of work-up, fast, low cost, avoidance of clean-up procedure, as well as the significant reduction of organic solvents and energy requirements in agreement with the principles of the Green Chemistry.

The proposed method was applied to the analysis of mussel samples from Galicia aquaculture ponds. Although the triazines under study were not detected in the samples, the analysis of these compounds in mussels is of great interest in order to evaluate risks for human health and also to control the quality of the marine environment.

Acknowledgements

This work has been supported by Program of Consolidation and Structuring of Units of Competitive Investigation of the University System of Galicia (Xunta de Galicia) potentially cofounded by European Regional Development Fund (ERDF) in the frame of the operative Program of Galicia 2007-2013 (reference: GRC2013-047). N. Rodríguez-González also thanks the "Xunta de Galicia" for the concession of her award under the program "Campus del Mar".

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ANEXO I-VI

Validation of a matrix solid phase dispersion methodology for the determination of triazines herbicides in fish. *Advances in Food Analysis Research, Nova Science Publishers* (2015) Capítulo 8, 167-176.

In: Advances in Food Analysis Research
Editor: Anita Haynes

ISBN: 978-1-63483-783-5

Chapter 8

VALIDATION OF A MATRIX SOLID PHASE DISPERSION METHODOLOGY FOR THE DETERMINATION OF TRIAZINES HERBICIDES IN FISH

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ABSTRACT

A method using dual process columns of Matrix Solid Phase Dispersion (MSPD) and Solid Phase Extraction (SPE) has been validated for extracting and cleaning-up of nine triazine herbicides (ametryn, atrazine, cyanazine, prometryn, propazine, simazine, simetryn, terbuthylazine and terbutryn) in trout samples. For this purpose, freeze dried samples (0.2 g) were blended with 2 g of octadecylsilyl-derivatized silica (Envi^{TM}-18) and transferred into a SPE cartridge containing ENVI^{TM}-Carb II/SAX/PSA (0.5/0.5/0.5 g) as clean up co-sorbent. Then the dispersed sample was washed with 10 mL of *n*-hexane and triazines were eluted with 20 mL ethyl acetate and 5 mL acetonitrile. Finally the extract was concentrated to dryness, re-constituted with 1 mL methanol and injected into the HPLC-DAD system. Recoveries varied between 88 and 105% with associate standard deviations below 8.6%, meeting the requirements stipulated by European Union legislation. The main advantages of this methodology when compared with conventional methods of sample preparation to screen herbicides in fish matrices are easy of work-up, fast, cheap, avoidance of clean-up procedure, as well as the reduction of organic solvents and energy requirements in agreement with the principles of Green Chemistry.

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1. INTRODUCTION

Fish can be harvested from waters that are contaminated by environmental chemicals, which may accumulate in fish at levels that can cause human health problems. Concern for these contaminants primarily focuses on fish harvested from aquaculture ponds, freshwater bodies, estuaries, and near-shore coastal waters (areas subject to shoreside contaminant discharges), rather than from the open ocean [1]. Environmental contaminants, such as pesticides, may also accumulate in aquacultured fish through contaminated feed ingredients [2]; furthermore, certain pesticides are applied directly to the water in aquaculture ponds to control weeds and algae and to eliminate invertebrates.

Nowadays there is a wide public interest in aquaculture. In fact, world aquaculture production is increasing much more rapidly than animal husbandry and capture fisheries, the other two sources of animal protein for the world population. There is widespread recognition that seafood production from capture fisheries is at or near its peak, and that aquaculture will become increasingly important as a source of seafood production [3]. Therefore the control of chemical groups which can originate important problems when reaching fish in farms is of a great interest.

Triazines are a group of herbicides that are present in the ten most-used herbicide formulations in Europe. Because of their relatively low soil adsorption and high solubility in water, these compounds migrate from soil to water [4] and due to the water cycle, fluxes of them reach seawater and affect the marine biota, having the coastal systems little capacity to degrade these compounds [5]. Furthermore, aquatic organisms are able to accumulate pesticide residues in much higher concentration than the surrounding water [6].

Therefore, triazines are considered as an important class of chemical pollutants and the Environmental Protection Agency (EPA) has proposed simazine and atrazine in the list of 67 pesticide for screening in the Endocrine Disruption Screening Program [7]. European Union has also included simazine and atrazine in the list of 33 priority substances in the European Union Water Framework Directive (2000/60/EC) [8], which committees the member states to achieve good qualitative and quantitative status of all water bodies, by way of Decision 2455/2001/EC [9]. Moreover, the Directive 2008/105/EC sets the Environmental Quality Standards (EQS) for these compounds in water and also shows the need to set EQS for these compounds in sediments and/or biota in order to protect the aquatic environment [10]. Since article 16 of the Water Framework Directive (2000/60/EC), demands a regular review of the priority substances, in January 2012, fifteen additional substances were proposed by the Commission (Proposal COM (2011)876) to be added to the list of priority substances [11]. Among these, terbutryn was proposed. European Union has not established yet limit values for triazines in fish and fishery products; however the U.S. Food and Drug Administration (FDA) has set a tolerance level of 12 mg kg^{-1} ww (wet weight) for simazine in the edible portion of finfish [1].

The sample preparation procedure is one of the most critical steps in analytical methods. In recent years, many innovations have been developed in the analytical processes applied to prepare food samples for the extraction and determination of pesticide residues. These methodologies include matrix solid phase-dispersion (MSPD), which combines aspects of several analytical techniques allowing sample homogenization, disruption, extraction, fractionation and clean-up within a single process [12]. In MSDP the solid sample is blended

in a mortar with an appropriate sorbent to obtain complete disruption and dispersion of the sample on the solid support. The blend is packed into a column from which the analytes are eluted with a relatively low solvent volume. Often, a co-sorbent material is placed at the bottom of the column to be filled with the blended sample to assist the extract clean up [13]. The key factors for the success of MSPD are its feasibility, flexibility, versatility, high throughput, low cost and rapidity. MSPD methods have been developed for the extraction of triazine residues from different plants and plant materials [14, 15]; however references for the determination of triazines in animal tissues by MSPD are scarce and to the best of our knowledge, no studies using MSPD have been done to extract these chemicals residues from finfish.

Therefore, the aim of this work was the validation of an effective, simple and fast method for the simultaneous determination of nine triazine herbicides in fish based on Matrix Solid Phase Dispersion (MSPD) and Solid Phase Extraction (SPE) clean-up followed by High Performance Liquid Chromatography (HPLC) coupled to Diode array Detection (DAD). In this study, rainbow trout (*Oncorhynchus mykiss*) from aquaculture was selected because there has been a large increase in salmonid production, and unlike many of the major products from aquaculture, there is substantially more aquaculture production of salmonids in developed countries than in developing countries [16]. On the other hand, trout is one of the four typical representative commodities included in the category of fish to carry out procedures for pesticide residues analysis in food and feed by SANCO guidelines [17]; furthermore, FDA considers the aquaculture trout as a potential fish-related hazard by environmental chemicals [1].

2. Material and Methods

2.1. Samples

1 kg of rainbow trout (*Oncorhynchus mykiss*) from aquaculture were purchased from a local market in A Coruña city (Northwest of Spain). Skin, bones and inner organs were discarded and muscle tissues were chopped, homogenized and freeze-dried. Then samples were homogenized again grounding it to a fine powder by an electrical mill and finally they were stored in glass bottles out of light exposure until analysis. The water content was determined gravimetrically by weighing before and after lyophilisation.

2.2. Chemicals

(a) *Herbicide standards* – Herbicides (ametryn, atrazine, cyanazine, prometryn, propazine, simazine, simetryn, terbuthylazine and terbutryn) analytical standards were supplied by Sigma-Aldrich (Inc. St. Louis, MO, USA). The individual stock standard solutions of 1000 mg L^{-1} were prepared in methanol by exact weighing of high-purity substances and stored at -18° C in the dark. Then a mixture of all the compounds was prepared in methanol containing 10 mg L^{-1} each individual triazine

and stored at -18° C. All working solutions were daily prepared by appropriate dilution of the 10 mg L^{-1} standard solutions with methanol.

(b) *Solvents* – *n*-hexane 95% and methanol were superpurity Solvents from Romil (Cambridge, UK). Acetonitrile (ACN) (HPLC grade) and ethyl acetate (PAR, solvents for analysis of pesticide residues by GC) for instrumental analysis were from Panreac (Barcelona, Spain). Milli-Q water was obtained from a purification system from Millipore (Billerica, MA).

(c) *Sorbents – Bulk packing:* Supelclean™ Envi™-18 and *SPE tubes*: Supelclean™ Envi-Carb™ II/SAX/PSA (500 mg/500 mg/500 mg) were from Sigma-Aldrich (Inc. St Louis, MO, USA).

(d) *Filters* – Polytetrafluoroethylene (PTFE) filters of 0.45 μm were from Teknocroma (Barcelona, Spain).

2.3. Materials and Apparatus

A Visiprep® vacuum distribution manifold from Supelco (Bellefonte, PA, USA) was employed in the purification step. A Büchi R-3000 rotary evaporator (Büchi Labortechnic AG, Flawil, Switzerland) was used in the evaporation step.

Chromatographic analyses were carried out in a high performance liquid chromatography-diode array detector (HPLC-DAD). The system consisted of a 2695 pump with a 996 Diode Array Detector from Waters (Milford, MA, USA). The column was a stainless steel column (150 mm x 4.6 mm ID, particle size 5 μm) packed with Hypersil GOLD C_{18} chemical bonded phase from Thermo Scientific (Austin, TX, USA).

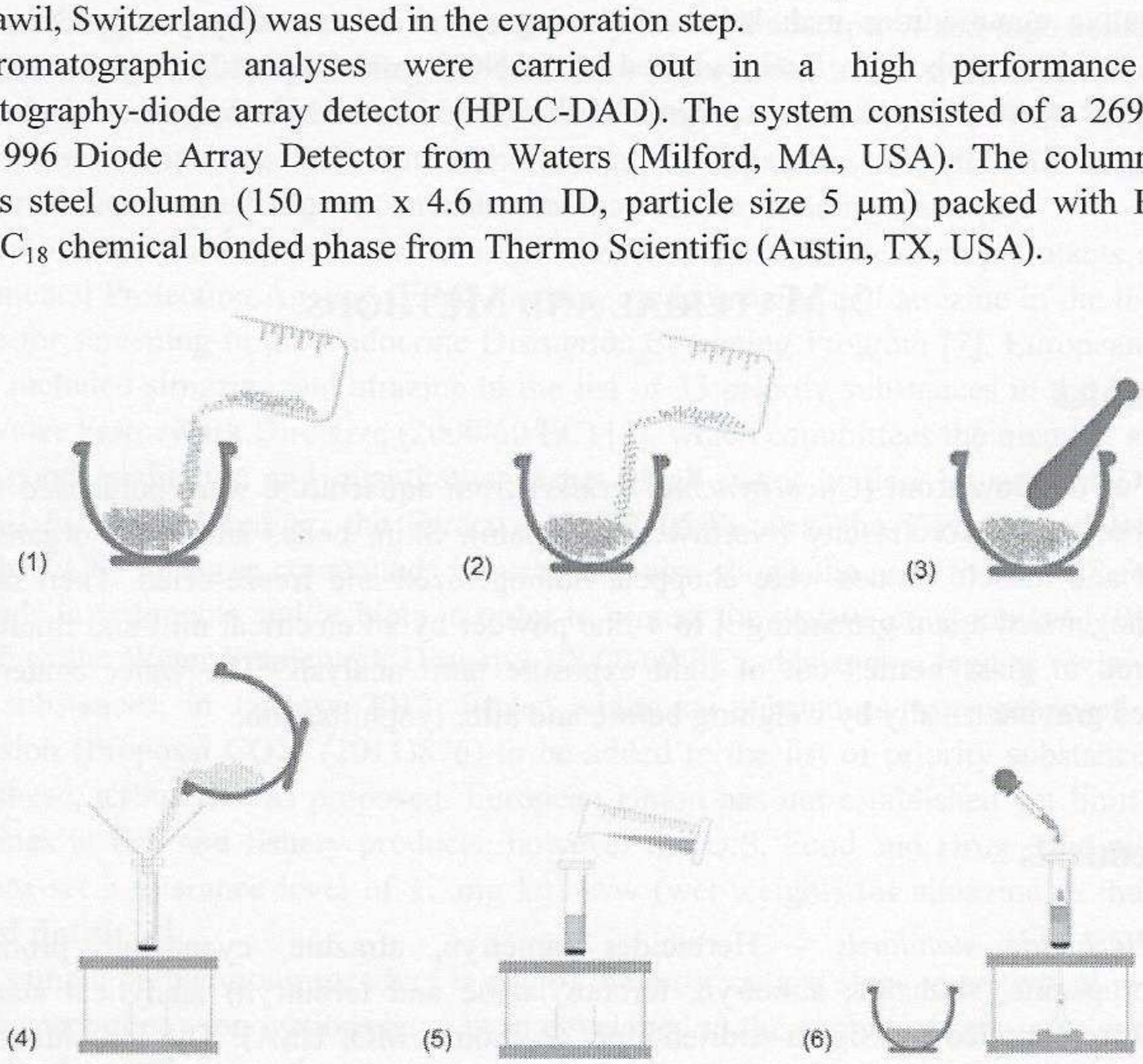


Figure 1. Scheme of the conditions of MSPD-SPE procedure.
(1) 2.00 g C18; (2) 0.2000 g freeze-dried trout; (3) Homogenization; (4) Transfer to a clean-up co column with EnviCarb/SAX/PSA (500/500/500mg); (5) Rinsing with 10 mL hexane; (6) Elution with 20 mL ethyl acetate and 5 mL acetonitrile.

2.4. Extraction Procedure

Samples were extracted by following a procedure previously developed in our laboratory for determination of the target compounds in mussel samples [18], with a modification regarding the sample amount. Under final conditions, 0.2000 g of freeze-dried trout sample was homogenised with 2.00 g of Envi^{TM}-18 in a glass mortar with a pestle for 5 min. The final mixture was transferred into a 20-mL SPE cartridge containing a triple sorbent layer of 1.5g Supelclean^{TM} Envi-Carb^{TM}-II/SAX/PSA (500/500/500 mg). Once packed, MSPD/SPE columns were connected to a Visiprep® vacuum distribution manifold and washed with 10 mL of hexane. Elution was performed with 20 mL of ethyl acetate and 5 mL of ACN and the obtained eluate was evaporated to a drop in rotary-evaporator and got to dryness by a gentle nitrogen stream. The residue was reconstituted in 1 mL methanol and the solution was filtered through of a 0.45 μm syringe filter of PTFE. As an illustration, Figure 1 shows the scheme of the MSPD-SPE procedure.

2.5. HPLC-DAD Conditions

The chromatographic analysis was carried out using the following ACN:H_2O gradient elution: ACN initial percentage of 30% (8 min), increased linearly to 40% in 5 min; increased to 50% in 5 min, after which the percentage was returned to the initial conditions in 9 min. A constant mobile phase flow rate of 1 mL min^{-1} and 20 μL of sample volume were used.

The absorbance was measured continuously in the 200-400 nm range and peaks areas quantification were carried out at 222.7 nm in order to achieve maximum sensitivity. All triazine herbicides were identified initially by retention time and then by spectral identification contrasting the spectrum with a standard library created in the same wavelength interval.

3. Results and Discussion

3.1. MSPD Application

Sorbent selection is of utmost importance since it is one of the variables controlling the selectivity of MSPD processes [19, 20]. In this work, we applied a method which employs C18 as dispersant, Envi-Carb II/SAX/PSA as clean-up co-sorbent and a mixture of ethyl acetate and acetonitrile as elution solvent. C18 is by far the most popular sorbent, especially for analyte extraction from animal. Regarding the tri-layer SPE cartridge, ENVI-Carb II is a graphitized non porous carbon that has a strong affinity towards planar molecules and can remove pigments and sterols, PSA is a polymerically bonded ethylenediamine-N-propyl phase that contains both primary and secondary amines, that retains fatty acids, organic acids, sugars and some polar pigments, and SAX is a quaternary amine that offers additional ion exchange capacity for removing matrix components.

During extraction of complex matrices, multiple unwanted compounds from the matrix are extracted along with the compounds of interest. A major issue when dealing with the

chromatographic determination of organic compounds in fish samples is to minimize the lipid content of the extracts [21, 22]. If lipids are not removed in the sample preparation process, they may cause retention time shifts, analyte peak distortion, decrease of sensitivity and reduction of column life, thus compromising quality of analysis. Therefore the determination the pesticides in food is often complicated by the presence of different fat content [23].

First assays were carried out employing 0.5000 g of trout sample following the procedure previously described for determination of triazines in mussel samples [18]. After evaporation of the eluates, it could be seen that the extracts had an appreciable amount of lipid material, and were not analysed further. This can be explained because of the higher lipid content in trout (4.8%) than in mussel (2.2%) [24]. Therefore, the following experiments were carried out using 0.2500 g of trout sample. In this case, after evaporation of eluates, extracts were clean enough to be processed; however after redissolution on methanol and filtration through PTFE filters, it could be observed some small particles of fat, thus the extracts were not analysed. Finally, considering 0.2000 g of trout sample, the extracts obtained were clean enough to be injected into the HPLC system. As an example, Figure 2 shows the chromatogram corresponding to a trout sample extracted and purified under the considered procedure; as it can be seen, none of the triazines under study were detected.

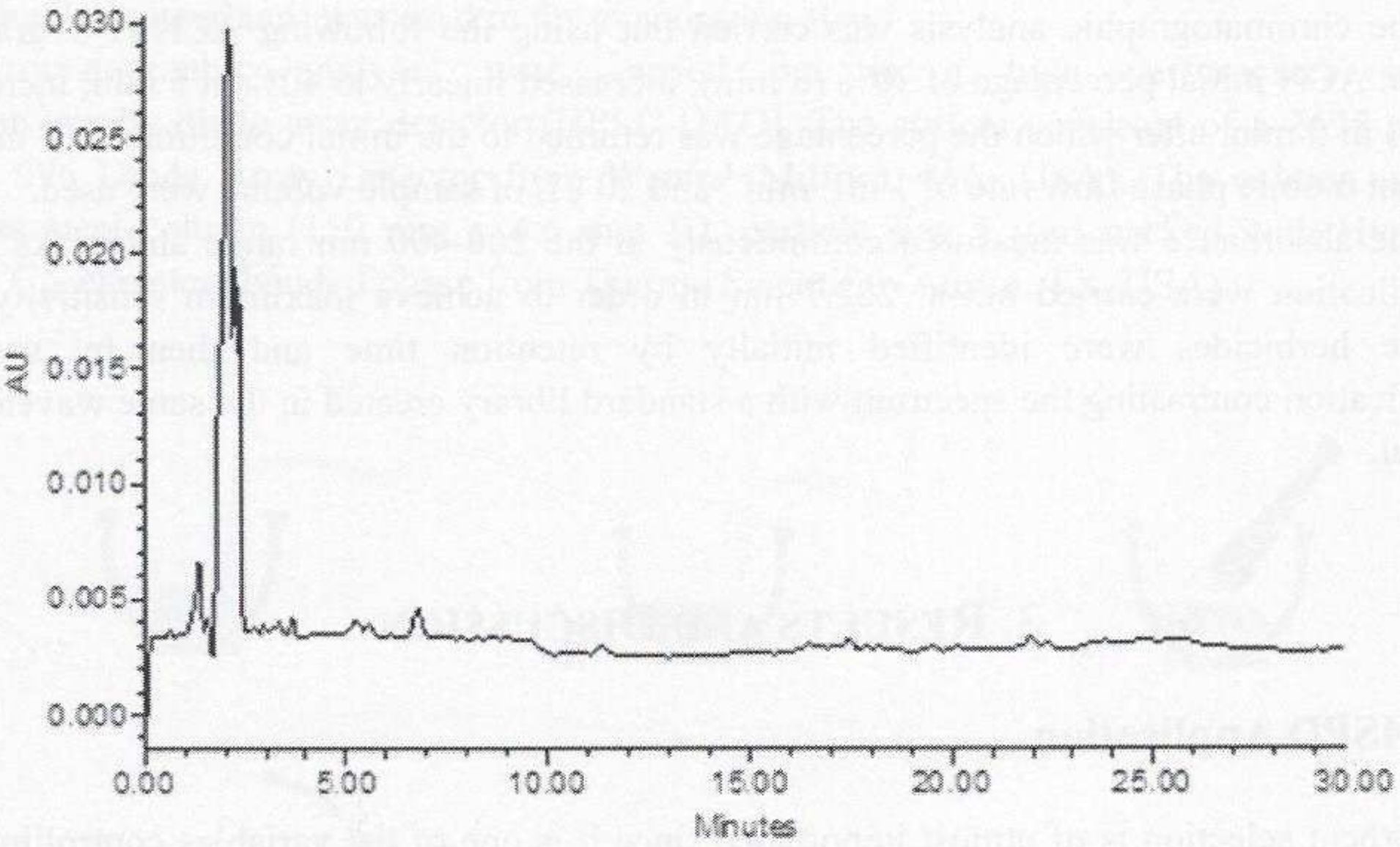


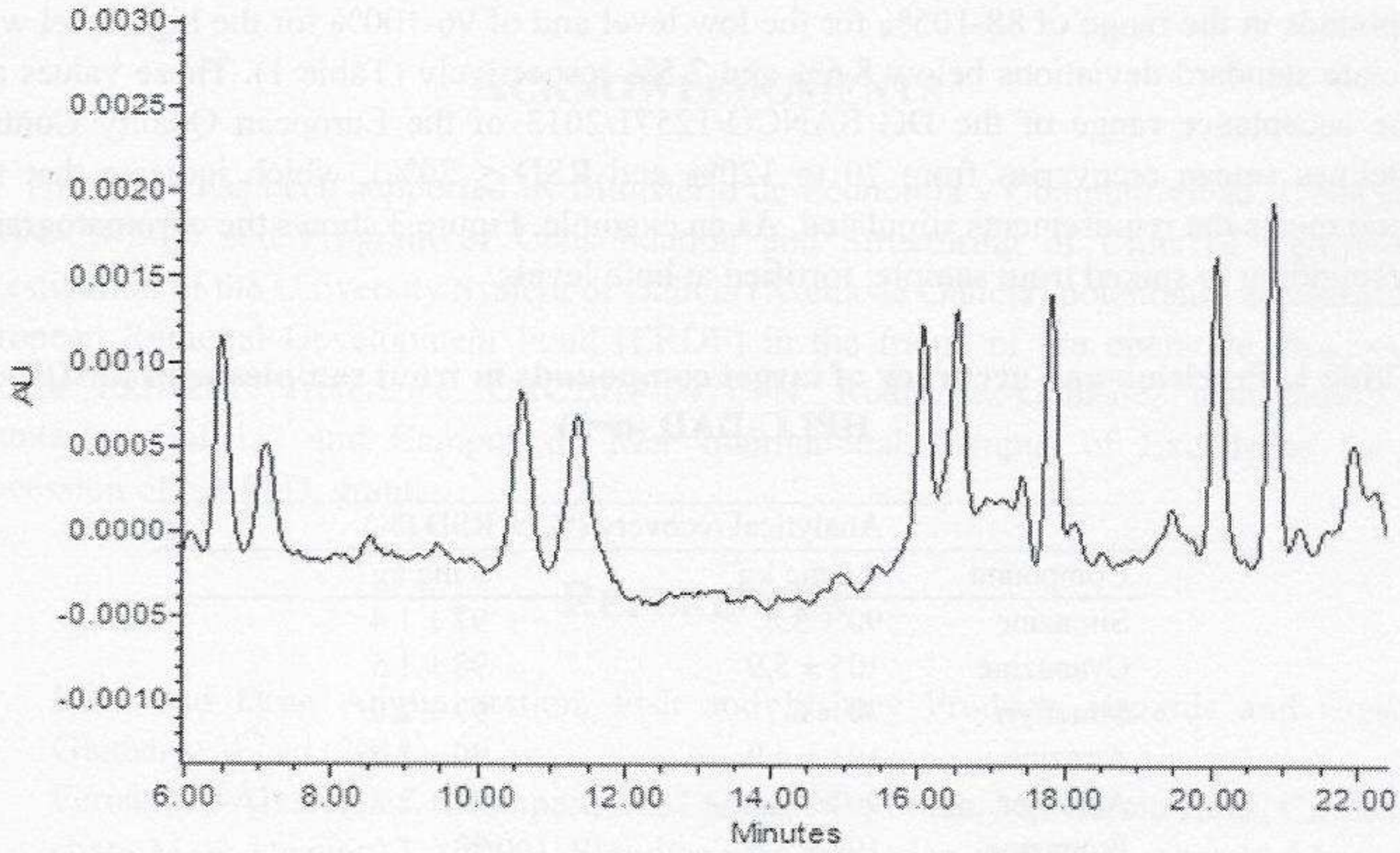
Figure 2. HPLC-UV chromatogram of a trout extract obtained by MSPD.

3.2. Method Validation

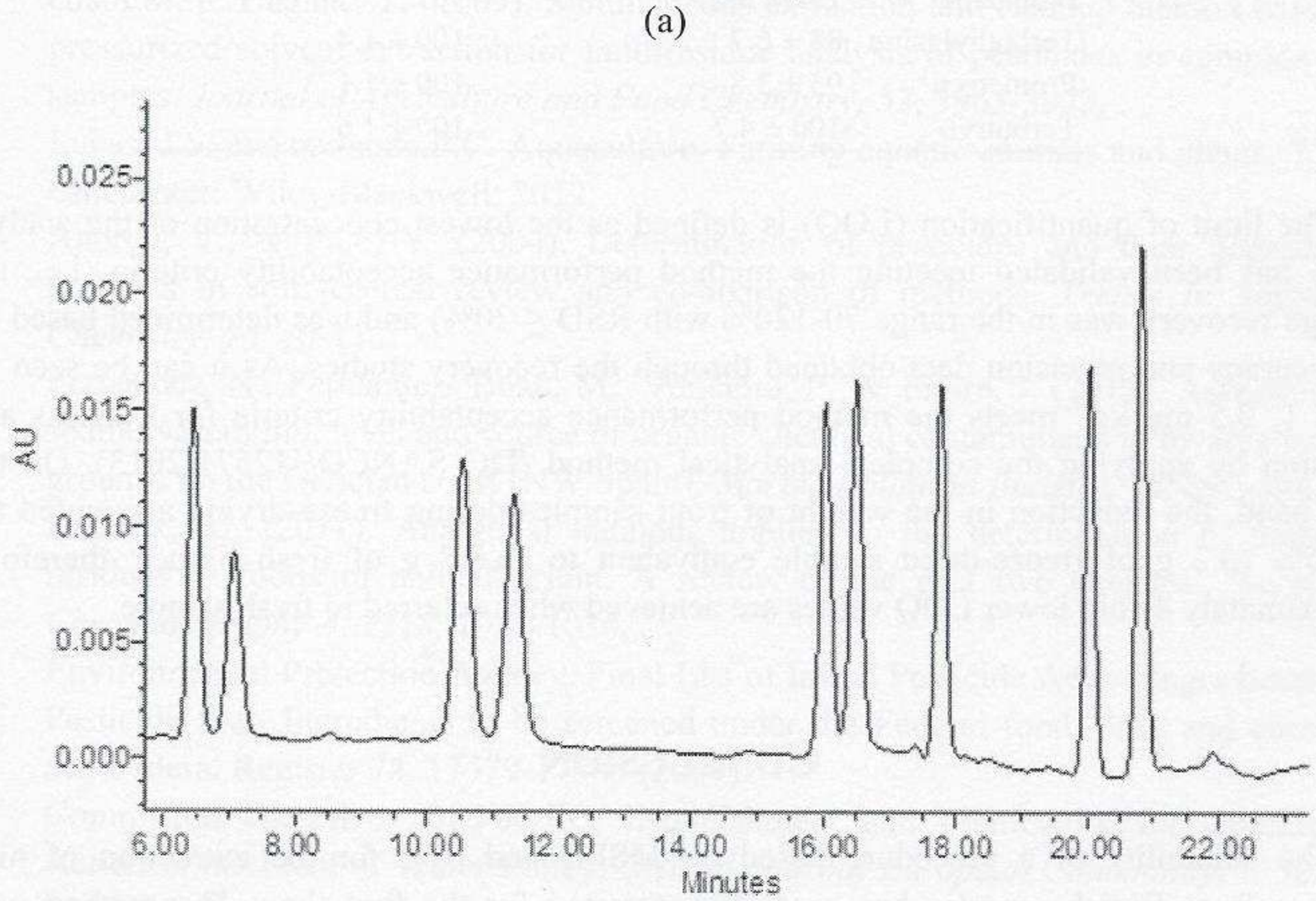
The method was validated in terms of accuracy, precision and limits of quantification according to "validation parameters and criteria" from SANCO Guidelines for method validation and quality control procedures for pesticide residue analysis in food and feed [17].

Quantitative results were calculated using matrix matched standards prepared by spiking the final extracts from blank samples of trout samples with different levels of triazines. The linearity of the calibration curves was calculated at a concentration range between 0.5-5 mg

kg^{-1} dried sample by duplicate analysis at five different concentration levels. Excellent linearities were obtained with coefficients of determination (R^2) higher than 0.998 for all triazines.



(a)



b)

Figure 3. HPLC-UV chromatograms of a spiked trout sample obtained by MSPD. (a) Trout sample spiked at 0.5 mg kg^{-1}; (b) trout sample spiked at 5 mg kg^{-1}. Target compounds are numbered as follows: (1) Simazine, (2) Cyanazine, (3) Simetryn, (4) Atrazine, (5) Ametryn, (6) Propazine, (7) Terbuthylazine, (8) Prometryn, (9) Terbutryn.

The accuracy and precision of the proposed method were determined by analysis of five replicates of uncontaminated trout sample spiked at two concentration levels (0.500 and 5.00 mg kg^{-1} dried sample, equivalent to 0.125 and 1.25 mg kg^{-1} fresh sample, respectively). The obtained results demonstrated that the method achieved satisfactory recoveries for all compounds in the range of 88-105% for the low level and of 96-100% for the high level with associate standard deviations below 8.6% and 2.5% respectively (Table 1). These values are in the acceptance range of the DG SANCO/12571/2013 of the European Quality Control Guidelines (mean recoveries from 70 to 120% and RSD $\leq$ 20%), which indicate that the method meets the requirements stipulated. As an example, Figure 3 shows the chromatograms corresponding to spiked trout sample, fortified at both levels.

Table 1. Precision and accuracy of target compounds in trout samples with MSDP-HPLC-DAD (n=5)

	Analytical recovery (%) ± RSD (%)	
Compound	0.5 mg kg^{-1}	5 mg kg^{-1}
Simazine	90 ± 3.8	97 ± 1.4
Cyanazine	105 ± 5.9	98 ± 1.5
Simetryn	90 ± 6.7	97 ± 2.0
Atrazine	101 ± 4.9	99 ± 2.5
Ametryn	96 ± 4.5	96 ± 2.3
Propazine	101 ± 8.6	98 ± 1.6
Terbuthylazine	88 ± 6.7	100 ± 1.4
Prometryn	95 ± 3.8	100 ± 1.1
Terbutryn	100 ± 4.7	100 ± 1.6

The limit of quantification (LOQ) is defined as the lowest concentration of the analyte which has been validated meeting the method performance acceptability criteria (i.e., the average recovery was in the range 70-120% with RSD $\leq$ 20%) and was determined based on the accuracy and precision data obtained through the recovery studies. As it can be seen on Table 1, 0.5 mg kg^{-1} meets the method performance acceptability criteria for trueness and precision by applying the complete analytical method (DG SANCO/ 12571/2013). On the other hand, the reduction in the weight of trout samples during freeze-drying accounted for ca. 75% (0.2 g of freeze-dried sample equivalent to ca.0.8 g of fresh tissue); therefore, approximately 4-fold lower LOQ values are achieved when referred to fresh sample.

CONCLUSION

The suitability of a procedure based on MSPD and SPE for the extraction of nine triazines from finfish samples has been demonstrated for the first time. The method uses Envi^{TM}-18 as dispersant with Envi-Carb^{TM} II/SAX/PSA co-column followed by a combination of 20 mL ethyl acetate and 5 mL acetonitrile as elution solvent. The developed method provides satisfactory accuracy and precision for the determination of triazines in aquaculture trout samples. The main advantages of this methodology when compared with classical methods of sample preparation to determine triazines in animal tissues are easy of

work-up, fast, low cost, avoidance of clean-up procedure, as well as the significant reduction of organic solvents and energy requirements in agreement with the principles of the Green Chemistry.

ACKNOWLEDGMENTS

This work has been supported by Ministerio de Economía y Competitividad (CTM2013-48194-C3-2-R) and Program of Consolidation and Structuring of Units of Competitive Investigation of the University System of Galicia (Xunta de Galicia) potentially cofounded by European Regional Development Fund (ERDF) in the frame of the operative Program of Galicia 2007-2013 (reference: GRC2013-047). N. Rodríguez-González also thanks the "Xunta de Galicia" and Campus do Mar International Campus of Excellence for the concession of her PhD. grant.

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ANEXO I-VII

Optimization of a HPLC-UV method for the analysis of Chloro-s-triazines in seawater samples. *Current Topics in Analytical Chemistry* (2016) 10, 23-28.

Current Topics in Analytical Chemistry

Original Communication

Optimization of a HPLC-UV method for the analysis of chloro-*s*-triazines in seawater samples

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ABSTRACT

A student activity that focuses on the determination of four triazines (atrazine, propazine, simazine and terbuthylazine) in seawater samples using High Performance Liquid Chromatography with Ultraviolet Detection (HPLC-UV) is presented. During the laboratory sessions students learn both the use of the instrument and the principles of chromatographic measurements by testing several mobile phases and applying the optimized method to the analysis of surface waters. Additionally, this project allows students to practice and develop a number of skills including solution preparation, solid phase extraction (SPE) and sample concentration procedures. This laboratory experiment is suitable for both analytical chemistry and environmental sciences and the use of this method as an undergraduate experiment permits to introduce concepts of green chemistry.

KEYWORDS: undergraduate laboratory instruction, analytical chemistry, HPLC, hands-on learning/ manipulatives, environmental science, triazines

1. INTRODUCTION

Triazines are well known herbicides that are applied worldwide to soil for the control of weeds in many agricultural fields, as well as, railway lines, roadsides and golf courses. Because of their relatively low soil adsorption and high solubility in water, these compounds migrate from soil to water [1] and as a result of the water cycle, fluxes of these compounds reach seawater. As the coastal systems have little capacity to degrade these compounds [2], biota from marine environment is affected [3, 4].

The mechanism of action of triazines is *via* photosynthetic inhibition, and for this reason, they are only toxic for plants; however these compounds can affect human health through the dietary intake. These compounds are highly persistent and can survive many years in soil, water and organisms. Therefore, they are considered as an important class of chemical pollutants, and the U.S. Environmental Protection Agency (EPA) has included simazine and atrazine in the list of 67 pesticides identified for the Endocrine Disruption Screening Program [5]. European Union has also included simazine and atrazine in the list of 33 priority substances covered under the EU Water Framework Directive [6], which requires the member states to achieve good qualitative and quantitative status for all water bodies, by way of Decision 2455/2001/EC [7]. Furthermore, the presence of pesticides in surface waters is regulated by the European Directive 2008/105/EC [8] that establishes a maximum permitted concentration of 2 and 4 μg L^{-1} for atrazine and simazine, respectively.

The determination of triazines is currently carried out by chromatographic techniques, mainly by Reversed Phase High Performance Liquid Chromatography (RP-HPLC), generally employing the mixture acetonitrile-water as mobile phase.

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Regarding the detection system, the detectors used are ultraviolet [9, 10], diode array [11, 12] or mass spectrometry [13]. For this project we have chosen UV detector because it is relatively inexpensive and also commonly found in teaching labs.

High performance liquid chromatography is an integral part of any analytical chemistry unit; in our laboratory there is a strong focus on HPLC as it is a technique commonly valued and looked for in our graduates when they pursue for academic and/or industry positions. Therefore, the present experiment has been designed to show the applications of reversed-phase chromatography and to train students in the use of high performance liquid chromatography by optimizing HPLC-RP methods and analyzing the presence of triazines in sea water samples. Chromatographic techniques are very important in analytical chemistry and environmental sciences; thus the project described is meant to be used as a demonstration whereby the students can understand the overlap among different fields of chemistry.

On the other hand, an extraction procedure to preconcentrate the analytes and remove possible interferences is mandatory to achieve the maximum permitted levels established for these pollutants in seawater samples by the European Directive. For this purpose, we have chosen solid phase extraction (SPE) because it is the preconcentration technique most commonly used for the determination of triazines in water samples [13, 14, 15].

Therefore the main aim of this work is the employment of a simple, effective and low-cost method for the determination of four triazine herbicides in seawater samples based on SPE followed by HPLC-UV as a laboratory experiment for undergraduate students. Thus this experiment acquaints students on a number of essential techniques in the analytical chemistry laboratory. Furthermore, the students would get hands-on experience on laboratory safety procedures and on waste disposal.

2. MATERIALS AND METHODS

2.1. Activity schedule

Before performing the laboratory sessions, students are required to complete a prelab exercise that assesses their preparedness to perform the experiment. They should to be able to describe the configuration of a HPLC system and what experimental parameters are expected to be important [16, 17]. The prelab exercise must be shown to the instructor at the beginning of the laboratory sessions.

The experiment is divided into two 3-h lab periods and one 2-h classroom period. In the laboratory sessions the students work as a group of six which allows to generate stimulating discussions between the members of the group. In the first lab period, students get acquainted with the HPLC technique (components of HPLC system and developing of HPLC-UV methods). In the second lab period they prepare the seawater samples by SPE and the standard solutions for calibrations, and then run the equipment to measure the concentration of triazines. Once completing the laboratory periods the students are required to make a report on the obtained results. Finally, a classroom session with all groups of students is carried out for a postlab discussion about the whole procedure.

2.2. Chemicals and materials

Herbicide analytical standards (atrazine, propazine, simazine and terbuthylazine) were supplied by Sigma-Aldrich. A solution containing all the studied triazines at a concentration of 10 μg mL^{-1} in methanol was prepared previously by the instructor. For quantitation of the target analytes linear calibration curves over 5 concentration levels (0.4, 0.6, 0.8, 1 and 2 μg mL^{-1}) were prepared. Acetonitrile (ACN) was purchased from Panreac and methanol and acetone from Romil. Ultra-pure Milli-Q water was obtained *via* a Millipore Milli-Q system (Millipore). Reverse phase polymeric cartridges Oasis HLB (6 mL, 200 mg) were supplied by Waters.

2.3. Hazards

Triazines (atrazine, simazine, propazine and terbuthylazine) are irritants to skin, eye and respiratory tract. Therefore, when handling them dispersion of dust should be avoided. Acetonitrile and methanol are highly flammable and can cause skin and eye irritation. They must be kept away from sources of ignition, heat surfaces and strong oxidizers. While using all these substances personal

protective equipments, such as gloves and goggles, must be worn at all times.

2.4. Chromatographic procedure

The HPLC equipment consisted of 1100 Agilent chromatograph equipped with a pump, an ultraviolet detector, a 20 µL Rheodyne injection loop and a computer that runs on ChemStation data processor. For separation an Envirosep PP column (125 mm x 4.6 mm ID, particle size 5 µm) from Phenomenex was employed.

Isocratic elution was performed with acetonitrile:water at several volume ratios (70:30, 55:45 and 45:55). The mobile phase flow rate was 0.8 mL/min in the three eluting regimes. Solvents were desgassed by a low flow of helium. The absorbance was measured at 223 nm in order to achieve maximum sensitivity.

2.5. Solid phase extraction procedure

Samples were extracted by following a procedure previously developed in our laboratory for the determination of the target compounds in seawater samples [15]. In summary it consists of the following steps: Oasis HLB cartridges were connected to a Visiprep® vacuum distribution manifold (Supelco). Prior to their use cartridges were conditioned by washing with 10 mL methanol and 10 mL Milli-Q water. Seawater sample (50 mL) was pumped through the cartridge at a flow rate of 10 mL min^{-1} and then the cartridges were washed with 20 mL Milli-Q water. Once the retention step had been completed, the cartridge was partially dried under a vacuum system for 5 min and totally dried using a nitrogen stream for 30 min. The elution of retained compounds was done with 3 mL of acetone and the organic extract was brought to complete dryness through a combination of rotary evaporator (Büchi, Labortechnic AG) at 40 ºC and a gentle nitrogen stream. Finally the sample was reconstituted in methanol/water (1:1, v/v) to a final volume of 1 mL and injected into the HPLC.

2.6. Treatment of residues

All solutions and HPLC eluent were discarded in an organic waste container. Gloves and tips from micropipettes were collected in a plastic residue container. Pasteur pipettes were disposed in a glass residue container.

3. RESULTS AND DISCUSSION

3.1. Optimization of the HPLC method

The triazine structure consists of a six-membered aromatic heterocyclic containing three nitrogens in the ring. The triazine herbicides currently used are mostly *1,3,5*-triazines (symmetrical triazines) which have a chlorine, thiomethyl or methoxy group at the 2-position of the ring and are usually substituted in the 4 and 6 positions with alkylamino groups [18].

For reversed phase partition mode the separation depends on the average hydrophilic or hydrophobic character of a compound. The components more soluble on the eluent are eluted earlier from the column. Triazines have both hydrophilic and hydrophobic functionalities spatially separated. The electron pairs on the N-atom rings form hydrogen bonds with water molecules and thus triazine rings are hydrophilic. However, the alkylamino chains in the 4 and 6 positions are hydrophobic [19]. The lipophilic/hydrophilic character of a substance is represented by octanol/ water partition coefficient (Kow) which is an important physicochemical descriptor for assessing transport pathways and environmental partitioning of herbicides [20]. In this work we determined four chloro-*s*-triazines whose structures and recommended log Kow [21, 22] are shown in figure 1. As it can be seen, the hydrophobicity increases with increasing length of the alkyl chains; therefore the elution order is simazine, atrazine, propazine and terbuthylazine.

The most widely used strategy for the development of a RP-HPLC method consists in starting at a high percentage of the organic component in the mobile phase and making stepwise changes in the solvent strength until the separation looks good. Then the percentage of each component in the mobile phase would be fine-tuned in smaller steps [23]. Therefore initially isocratic elution with a ratio 70% ACN:30% H_2O was carried out; at this ratio the peaks are distinguished but are not fully separated. At 55% ACN:45% H_2O the peaks of simazine and atrazine are adequately resolved but propazine and terbuthylazine remain partially overlapped. At 45% ACN:55% H_2O good resolution of the four triazines is achieved. Thus this elution ratio was chosen for the qualitative

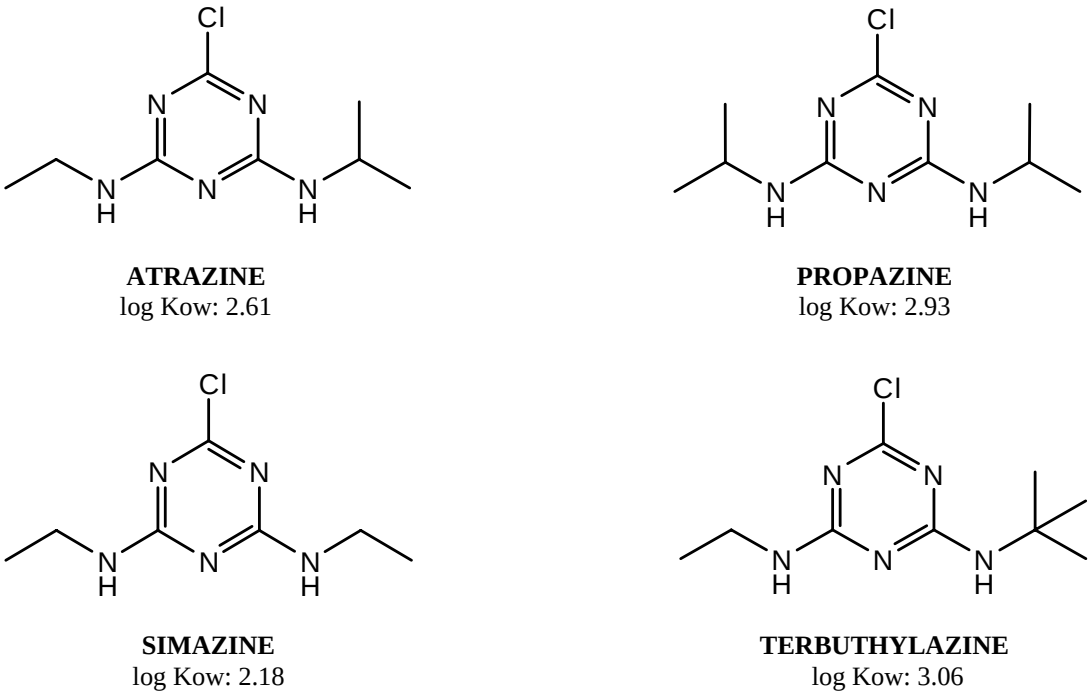

Figure 1. Structures and recommended log Kow of chloro-*s*-triazines.

and quantitative analysis of a surface water sample. Figure 2 shows the chromatograms obtained at the three elutions regimes. As it can be seen, three changes can be observed as a result of the decrease in solvent strength: longer retention times, the overall separation tends to improve and peaks become broader and shorter.

Once the optimal mobile phase was selected, the five standard solutions were injected. Calibration curves were constructed using least-squares regression of concentration versus the peak area of the calibrations standards. Then a seawater sample was analysed.

3.2. Laboratory experience for undergraduate students

Our main aim was to design a laboratory experiment that allows students to participate actively in the learning of concepts through practical experience. It increases the student motivation and makes teaching the principles involved in chromatographic techniques easier. On the other hand, future chemists must know the necessary tools to support and promote global sustainability [24, 25], which lead to safer and more efficient work environments on the laboratory. Therefore green chemistry principles have been incorporated at the laboratory classes. Furthermore, the procedure described herein is meant to be used as a demonstration whereby the students can understand the overlap among different fields of chemistry.

The four pedagogical goals of this experiment were: 1) Introduction to the HPLC-UV instrumentation (components of HPLC system and optimization of HPLC-RP methods); 2) Learning of SPE procedure as a sample pretreatment technique as well as the concentration step; 3) Qualitative and quantitative analysis; and 4) Evaluation of the process taking into account the principles of green chemistry (waste generation, safety of processing steps, health and environmental impact of the reagents, etc.). The experiment also opens the discussion of other concepts such as absorbance, chemical functionality (hydrophobibicity/philicity, toxicity, etc.), and state-of-art about legislation of triazines in sea water.

After finishing the laboratory sessions the students are required to make a report on the obtained results, which will help the students to develop the scientific writing skills they would

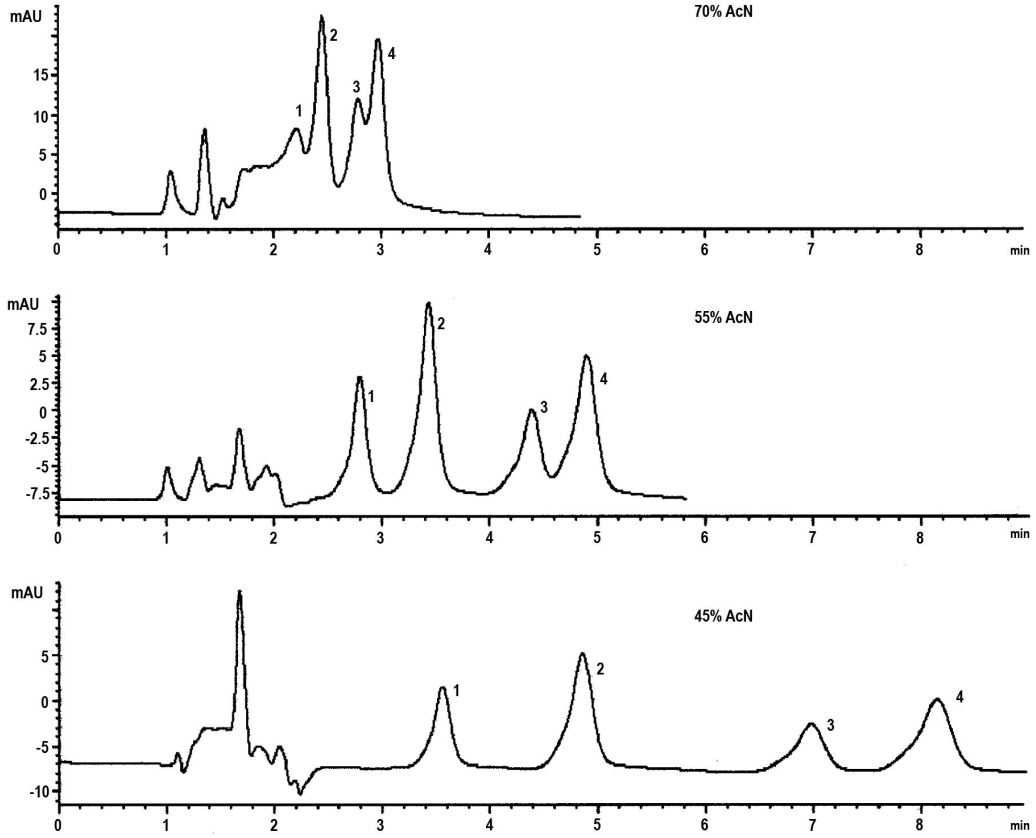

Figure 2. Chromatogram showing separation of triazines at three elution regimes. Compounds are numbered as follows: (1) Simazine, (2) Atrazine, (3) Propazine, and (4) Terbuthylazine.

need later in their careers. This report requires students to interpret and analyse their data and also to answer a list of questions on the RP-HPLC-UV technique.

Finally, after all students in the course have completed the laboratory periods, they are required to present their results in a classroom session. This will instigate interesting discussions because the different groups of students have to compare the results obtained to formulate explanations about the experimental procedure carried out, and also propose solutions to obstacles or undesirable outcomes. They also evaluate the whole procedure taking into account the principles of green chemistry.

4. CONCLUSION

This project is suitable for a typical undergraduate analytical laboratory course and provides students a good opportunity to understand the connection between analytical chemistry and environmental sciences. The experiment is mainly focused to train students in the use of high performance liquid chromatography by optimizing HPLC-RP methods. Additionally this activity acquaints students on preparation of solutions, solid phase-extraction of herbicide residues from liquid samples and employment of concentration steps. Therefore it permits the students to gain experience on a number of essential techniques in the analytical chemistry laboratory. Furthermore they also get hands-on experience on laboratory safety procedures and familiarity with some of the principles of green chemistry. This activity is simple, cost-effective, and only requires readily available apparatus.

ACKNOWLEDGMENTS

This work was financially supported by Xunta de Galicia potentially co-funded by European Regional Development Fund (EDRF) and by the Spanish Ministry of Economy, and Competitiveness

co-funded by ERDF (CTM2013-48194-C3-2-R). N. Rodríguez-González also thanks the "Xunta de Galicia" for the concession of her Ph.D grant under the program of the International Campus of Excellence "Campus del Mar".

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest

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ANEXO II

CONGRESOS

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ANEXO II-I

Simultaneous determination of nine triazines in seawater by solid-phase extraction followed by high performance liquid chromatography-diode array detector. *"7th European Conference on Pesticides and Related Organic Micropollutants in the Environment" and "13th Symposium on Chemistry and Fate of Modern Pesticides"*. Porto (2012). (ISBN: 978-989-20-3262-7).

October 7-10, 2012
Porto, Portugal

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7th European Conference on Pesticides and Related Organic Micropollutants in the Environment

13th Symposium on Chemistry and Fate of Modern Pesticides

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Session IA: P14

SIMULTANEOUS DETERMINATION OF NINE TRIAZINES IN SEAWATER BY SOLID-PHASE EXTRACTION FOLLOWED BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-DIODE ARRAY DETECTOR

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Summary

In this work, two high performance liquid chromatographic methods for the determination of nine triazine herbicides (ametryn, atrazine, cyanazine, prometryn, propazine, simazine, simetryn, terbuthylazine and terbutryn) in water samples were compared. For this purpose, a method using isocratic elution with methanol-water as mobile phase was developed and compared with a method using acetonitrile-water and gradient elution. A procedure based on solid phase extraction employing Oasis HLB cartridges was optimized using 500 mL of sample prior to the chromatographic determination.

The SPE-HPLC method using acetonitrile-water as mobile phase was successfully applied to the determination of triazines in seawater. The analytical characteristics using 50 mL of sample volume were evaluated. Finally, this method was applied to the analysis of seawater samples from Vigo and Isla de Arosa estuaries (Galicia, NW Spain).

Introduction

The high persistence of pesticides has required rigorous control of environmental contamination. Therefore, the presence of pesticides in surface waters is regulated by the European Directive 2008/105/EC [1] that establishes 2 and 4 $\mu g L^{-1}$ as alert and alarm threshold values respectively. It is important to take into account that atrazine and simazine have been included in a list of "priority hazardous substances" (Decision n.o 2455/2001/EC [2], that amends the Directive 2000/60/EC [3]) due to their persistence, toxicity, moderate leaching capacity and potential to adsorb onto soils and sediments.

The aim of this work was the comparison of two mobile phases for the simultaneous determination of nine triazines in water by SPE-HPLC with DAD detection, as well as the application of the most suitable method to the analysis of seawater samples.

Results and Discussion

Chromatographic methods were compared by evaluating the limit of detection (LOD) and quantification (LOQ), linearity and precision. Results have shown lower detection and quantification limits for the method using acetonitrile-water as mobile phase (LODs varied from 4 to 15 $\mu g\ L^{-1}$ and 72 to 157 $\mu g\ L^{-1}$ for acetonitrile and methanol method respectively). LODs obtained were not adequate for the determination of these compounds at the levels requested by the 2008/105/EC Directive [1]. Therefore, a procedure based on solid phase extraction employing Oasis HLB cartridges using 500 mL of water was optimized prior to the chromatographic determination. The overall procedure was satisfactory in both cases, with quantification limits ranged from 0.032 to 0.069 $\mu g\ L^{-1}$ and from 0.52 to 1.79 $\mu g\ L^{-1}$ for acetonitrile and methanol method respectively. Furthermore, repeatability (2.4-7.6% for ACN method and 2.6-12% for MeOH method), intermediate precision (0.9-11.0% for ACN method and 1.5-6.9% for MeOH method) and recoveries (80-99% for ACN method and 83-103% for MeOH method) calculated at 2 $\mu g\ L^{-1}$ (legislation level) were shown to be adequate.

The SPE-HPLC method using acetonitrile-water was applied to the determination of triazines in seawater using 50 mL of sample volume. The study of linearity and sensitivity was carried out at 0.5-3 μg L^{-1} range, and repeatability and recoveries were calculated at 2 μg L^{-1} levels. The stability of herbicides stored on Oasis HLB cartridges was also studied. Detection limits were adequate (LODs varied from 0.15 to 0.38 μg L^{-1}) and recoveries obtained after two weeks of storage at – 20 ºC were satisfactory. None of the herbicides were detected in the 51 analyzed seawater samples from Vigo and Isla de Arosa estuaries.

Conclusions

An effective, accurate, simple and low-cost method based on SPE combined with HPLC-DAD for the simultaneous analysis of triazines in seawater was developed. It has to be noted that only a few papers have been published on SPE for triazines in seawater and, to the best of our knowledge, there is no any article which determine simultaneously nine triazines in seawater samples.

Acknowledgments

The authors would like to thank the financial support of the “Ministerio de Educación y Ciencia” (Project CTQ2007-63949/BQU) and the “Xunta de Galicia” (Project 09MDS038103PR). R. Pouso-Blanco and N. Rodríguez-González also thanks to the “Xunta de Galicia” and “Campus del mar” respectively, for the concession of their award.

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ANEXO II-II

On-line solid-phase extraction method for determination of triazine herbicides and degradation products in seawater by Ultra-Pressure Liquid Chromatography-Tandem Mass Spectrometry. *"9th European Conference on Pesticides and Related Organic Micropollutants in the Environment" and "15th Symposium on Chemistry and Fate of Modern Pesticides"*. Santiago (2016). (ISBN: 978-84-945958-1-3).

9th European Conference on Pesticides and Related Organic Micropollutants in the Environment

15th Symposium on Chemistry and Fate of Modern Pesticides

Santiago de Compostela, Spain
October 4th – 7th 2016

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International Association of Environmental Analytical Chemistry
GRIFA
European - Mediterranean Association ENVIRONMENTAL EDUCATION ASSESSMENT AND PROTECTION

ISBN: 978-84-945958-1-3
Dep. Legal: C 1708-2016

P8: ON-LINE SOLID-PHASE EXTRACTION METHOD FOR DETERMINATION OF TRIAZINE HERBICIDES AND DEGRADATION PRODUCTS IN SEAWATER BY ULTRA-PRESSURE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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SUMMARY

A fast, simple, selective and sensitive method has been developed for the determination of nine triazine herbicides (ametryn, atrazine, cyanazine, prometryn, propazine, simazine, simetryn, terbuthylazine and terbutryn) and eight degradation products (desethyl atrazine, desethyl-desisopropyl atrazine, desethyl 2-hydroxyatrazine, desethyl terbuthylazine, desisopropyl atrazine, desisopropyl 2-hydroxyatrazine, 2-hydroxyatrazine and 2-hidroxyterbuthylazine) in seawater samples. On-line solid-phase extraction coupled with ultra-pressure liquid chromatography-tandem mass spectrometry was employed for simultaneous analysis of all compounds in 11 min. Limits of quantification ranged from 0.023 to 0.657 μg L^{-1} permit to ensure proper determination at the levels established by the Directive 2013/39/UE. Good linearity was obtained for all compounds with $r^2 > 0.99$ in all cases. Furthermore, inter-day precision (0.0-2.1%), intra-day precision (0.0-3.9%) and recoveries (80.3-99.8%) were shown to be satisfactory. Finally, the proposed method was applied to the analysis of the target compounds in seawater samples from Porto (Portugal).

Keywords: Triazines; Degradation products; Seawater; Ultra-pressure liquid chromatography; Mass spectrometry

INTRODUCTION

The Directive 2013/39/EU calls the attention to the important role of monitoring emerging pollutants that are not regularly considered in monitoring programs but that can have toxicological effects [1]. This Directive includes three triazines (atrazine, simazine and terbutryn); however, the impact due to herbicides tends to be underestimated when only the triazines are analysed. Therefore, the main degradation products should be included in current analytical methods.

MATERIALS AND METHODS

An Acquity UPLC with an UPLC BEH C18 column and a triple-quadrupole mass spectrometer fitted with an electrospray ionization source was used. Gradient elution was performed adding ammonium acetate in both phases (water and methanol). The solid phase extraction was carried out using OASIS® HLB on-line SPE cartridge.

RESULTS AND DISCUSSION

UPLC-MS/MS conditions (cone voltage, collision energy, negative or positive mode, mobile phase composition and gradient elution) were optimized. The method allowed the detection of 17 pesticides in 11 min.

The SPE procedure was based on a previously developed method using Oasis HLB for triazines analysis [2] and taking into account that Oasis HLB has shown to have better ability to retain some degradation products than other sorbents [3].

The analytical characteristics of the developed method were satisfactory for all compounds. On-line SPE recoveries in spiked unpolluted seawater sample were calculated and acceptable values were found.
The method was applied to the analysis of the target compounds in seawater from the coastline of Porto. Although the compounds have not been detected, the monitoring of their levels in marine ecosystems is of great economic and environmental importance.

CONCLUSIONS

The on-line SPE-UPLC-MS/MS method developed enables the analysis of nine triazines and their main degradation products in seawater at the levels required by European Union legislation [1]; consequently, it can be an important tool to control the presence of these compounds in seawater samples. The method has shown suitable precision and good recovery values were obtained for all compounds. To best of our knowledge, no studies using on-line SPE-UPLC-MS/MS have been done to determine the target compounds in seawater. Furthermore, an important difference of the proposed method with previously described methodology for the analysis of triazines herbicides and their main degradation products is the determination of a greater number of degradation products simultaneously with triazines.

ACKNOWLEDGEMENTS

This work was supported by Ministerio de Economía y Competitividad (CTM2013-48194-C3-2-R) and Program of Consolidation and Structuring of Units of Competitive Investigation of the University System of Galicia (reference: GRC2013-047).

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ANEXO II-III

Development of a matrix solid phase dispersion methodology for the determination of triazine herbicides in marine sediments. *"9th European Conference on Pesticides and Related Organic Micropollutants in the Environment"* and *"15th Symposium on Chemistry and Fate of Modern Pesticides".* Santiago de Compostela (2016). (ISBN: 978-84-945958-1-3).

9th European Conference on Pesticides and Related Organic Micropollutants in the Environment

15th Symposium on Chemistry and Fate of Modern Pesticides

Santiago de Compostela, Spain
October 4th – 7th 2016

BOOK OF ABSTRACTS

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA
CRETUS
INGACAL INSTITUTO GALEGO DA CALIDADE ALIMENTARIA
XUNTA DE GALICIA
CONSELLERÍA DO MEDIO RURAL
CENTRO INVESTIGACIÓNS AGRARIAS MABEGONDO
International Association of Environmental Analytical Chemistry
GRIFA
European - Mediterranean Association ENVIRONMENTAL EDUCATION ASSESSMENT AND PROTECTION

ISBN: 978-84-945958-1-3
Dep. Legal: C 1708-2016

P2: DEVELOPMENT OF A MATRIX SOLID PHASE DISPERSION METHODOLOGY FOR THE DETERMINATION OF TRIAZINE HERBICIDES IN MARINE SEDIMENTS

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SUMMARY

A method based on Matrix Solid Phase Dispersion (MSPD) for the determination of nine triazines in marine sediments was optimized in terms of dispersants, co-sorbents and elution solvents. Three dispersing agents: ENVI-18, ENVI-Carb and diatomaceous earth, and four clean-up co-sorbents: ENVI-Florisil, ENVI-Carb, ENVI-Carb-II/PSA and SAX/PSA were assayed. Then extracts were concentrated to dryness, re-constituted with 1 mL methanol and determined by HPLC-DAD. The best results were obtained with ENVI-Carb as dispersing agent using 20 mL of ethyl acetate as elution solvent. The linearity of the calibration curves yielded the coefficients of determination $\geq$0.9992. The analytical recoveries obtained were close to 100% and repeatability and reproducibility were below than 3.5 % for all compounds. The LOQ values ranged from 0.022 to 0.037 mg kg^{-1}. Finally the method was applied to analyse the target compounds in marine sediment samples from the estuary of A Coruña (NW Spain). Results were confirmed by HPLC-MS/MS.

Key Words: Triazine herbicides. Marine sediments. Matrix solid phase dispersion.

INTRODUCTION

The contamination of the marine environment from herbicides is of great concern because of their high distribution in the aquatic system and of their toxic properties. Triazine herbicides are considered as an important class of chemical pollutants and European Union has included simazine and atrazine in the list of 33 priority substances in the EU Water Framework Directive (2000/60/EC) [1]. Moreover, the Directive 2008/105/EC [2] sets the Environmental Quality Standards (EQS) for these compounds in water and also shows the need to set EQS for these compounds in sediments and/or biota. Recently, terbutryn has been added to the list of priority substances by European Directive 2013/39/EU [3].

MATERIALS AND METHODS

Under final working conditions, 1.0000 g of freeze-dried sediment sample was homogenised with 1 g of ENVI-Carb in a glass mortar with a pestle for 5 min. The final mixture was transferred into a 6 mL glass syringe and once packed, MSPD columns were connected to a Visiprep® vacuum distribution manifold. Elution was performed with 20 mL of ethyl acetate and the obtained eluate was evaporated to a drop in rotary-evaporator and got to dryness by a gentle nitrogen stream. The residue was reconstituted in 1 mL methanol and then filtered through of a 0.45 µm syringe filter of PTFE.

RESULTS AND DISCUSSION

ENVI-18 as dispersant was tested with the four online clean-up co-sorbents. Experiments carried out using SAX/PSA and ENVI-Florisil led to yellow extracts, whereas ENVI- Carb and ENVI-Carb-II/PSA provided colourless eluates; satisfactory recoveries (>80%) for all triazines were achieved with the four systems assayed. Then, three dispersants without using clean-up co-sorbent were evaluated: ENVI-18, diatomaceous earth and ENVI-Carb; the efficiency of the

three dispersants in terms of recoveries was satisfactory for all triazines in all cases (>85%); on the other hand, ENVI-Carb was the only one that gave colourless eluates. The analytical features of the method (linearity, limits of quantification, trueness and precision) were satisfactory. The method was applied to investigate the presence of the target species in ten marine sediments from the estuary of A Coruña. None of them contained detectable amount of target herbicides.

CONCLUSIONS

The suitability of a procedure based on MSPD for the extraction of nine triazines from sediment samples has been demonstrated for the first time. The method uses ENVI-Carb as dipersant with 20 mL ethyl acetate as elution solvent. The developed method provides satisfactory trueness and precision for the determination of triazines in marine sediments.

ACKNOWLEDGEMENTS

This work has been financially supported by Xunta de Galicia potentially cofounded by European Regional Development Fund (EDRF) and by the Spanish Ministry of Economy and Competitiveness co-funded by ERDF (CTM2013-48194-C3-2-R). N. Rodríguez-González also thanks Xunta de Galicia for the concession of her Ph.D grant under the program of the International Campus of Excellence “Campus del Mar”.

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ANEXO II-IV

Determination of triazinic herbicides in seawater samples by high performance liquid chromatography. Rodríguez-González, N., Beceiro-González, E., González Castro, M.J. *XXXV Bienal RSEQ*. A Coruña (2015). (ISSN: 978-84-606-9786-2).

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Libro de Resúmenes

XXXV Reunión Bienal RSEQ

A Coruña

19 a 23 Julio 2015

RSEQ
Real Sociedad Española de Química

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www.bienal2015.cienciasudc.es/es/

Libro de resúmenes de la XXXV Reunión Bienal de la Real Sociedad Española de Química / Book of Abstracts of the XXXV Biennial Meeting of the Spanish Royal Society of Chemistry

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José Pérez Sestelo – Universidade da Coruña

Darío Prada Rodríguez – Universidade da Coruña

Publicado / Published: Julio 2015 / July 2015

ISBN: 978-84-606-9786-2

Cite como / Cite as:

Español:
Autor(es), *Libro de resúmenes*, XXXV Reunión Bienal de la Real Sociedad Española de Química, A Coruña (España), Julio 19-23, 2015; Editores: J. Arturo Santaballa López, Emilia Iglesias Martínez, Teresa Rodríguez Blas, Moisés Canle López, José Pérez Sestelo. Darío Prada Rodríguez; pág. página.

English:
Author(s), *Book of Abstracts*, XXXV Biennial Meeting of the Spanish Royal Society of Chemistry, A Coruña (Spain), Jul 19-23, 2015; J. Arturo Santaballa López, Emilia Iglesias Martínez, Teresa Rodríguez Blas, Moisés Canle López, José Pérez Sestelo. Darío Prada Rodríguez, Eds.; pp page.

S4: *Enseñanza, Historia y Divulgación de la Química*

DETERMINATION OF TRIAZINIC HERBICIDES IN SEAWATER SAMPLES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Keywords: *Undergraduate Laboratory Instruction, Analytical Chemistry, HPLC, Hands-On Learning/Manipulatives, Environmental Science, Triazines*

A student activity that focuses on the determination of four chloro-*s*-triazines (atrazine, propazine, simazine and terbuthylazine) in sea water samples by using High Performance Liquid Chromatography with Ultraviolet Detection (HPLC-UV) is presented. During the laboratory sessions, students learn both the use of the instrument and the principles of chromatographic measurements by testing several mobile phases and applying the optimized method to the analysis of surface waters. Additionally, this project has students practicing and developing a number of skills including solution preparation, solid phase extraction (SPE) and sample concentration procedures.

Before performing the laboratory period, students are required to complete a prelab exercise that assesses their preparedness to perform the experiment. They need to be able to describe the configuration of a HPLC system and what experimental parameters are expected to be important. The prelab exercise must be shown to the instructor at the beginning of the laboratory sessions.

The experiment is divided into two 3 h lab periods and one 2 h classroom period. In the laboratory sessions, the students work in small groups which let to generate stimulant discussions between the members of the group. In the first lab period, students acquaint with HPLC technique (components of HPLC system and development of HPLC-UV methods). In the second lab period, they prepare the seawater samples by SPE and the standard solutions for calibration; then run the equipment to measure the concentration of triazines. After completing the experiment, the students are required to pool their results in a report, which helps the students to develop the scientific writing skills they will need later in their careers. Finally, a classroom session with all groups of students is carried out for a postlab discussion about the overall procedure.

This project allows students to participate actively in learning concepts through practical experience. It increases the student motivation and makes teaching the principles involved in chromatographic techniques easier.

Acknowledgements: This work has been supported by Program of Consolidation and Structuring of Units of Competitive Investigation of the University System of Galicia (Xunta de Galicia) potentially cofounded by European Regional Development Fund (ERDF) (reference: GRC2013-047).